Studies on polar cell wall growth and antibiotic susceptibility of *Corynebacterium glutamicum*

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“If you can't explain it simply, you don't understand it well enough.”

Albert Einstein

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Martinsried, den 2. März 2015 Boris Sieger

CONTENTS

ABBREVIATIONS

A	Alanine
AA	Amino acid
AB	Antibiotic
AG	Arabinogalactan
BHI	Brain heart infusion complex growth medium
Bsu	*Bacillus subtilis*
BTZ	Benzothiazinone
Cgl	*Corynebacterium glutamicum*
D	Aspartate
DAP	Diaminopimelic acid
DBCO	Dibenzocyclooctyne
DHPE	Dihexadecanoyl-glycero-3-phosphoethanolamine
DNA	Deoxyribonucleic acid
DPA	Decaprenylphosphoryl-D-arabinofuranose
DprE1	Decaprenylphosphoryl-β-D-ribose 2'-epimerase
DprE2	Decaprenylphosphoryl-D-2-keto-erythro-pentose reductase
DPP	Decaprenyl pyrophosphate
DPPR	Decaprenylphosphoryl 5-phosphoribose
DPR	Decaprenylphosphoryl ribose
DPX	Decaprenylphosphoryl "oxoderivative"
EMB	Ethambutol
F	Phenylalanine
FRET	Förster (Fluorescence) Resonance Energy Transfer
G	Glycine
I	Isoleucine
INH	Isoniazid (Isonicotinylhydrazine)
K	Lysine
LAM	Lipoarabinomannan
LB	Luria Bertani complex growth medium

MA	Mycolic acid
MCS	Multiple cloning site
MS	Manuscript
Mtu	*Mycobacterium tuberculosis*
OD_{600}	Optical density (at a wavelength of 600 nm)
PBP	Penicillin binding protein
PG	Peptidoglycan
PRPP	Phosphoribosyl pyrophosphate
PS	Polysaccharide
Q	Glutamine
R	Arginine
S	Serine
STPK	Serine/threonine protein kinase
Tab.	Table
TB	Tuberculosis
UDP	Undecaprenyl pyrophosphate
WT	Wild type

ABSTRACT

Corynebacterium glutamicum is a Gram positive soil bacterium with high industrial importance in ton scale production of amino acids. Apart from that, it becomes more and more important for medical studies, where it serves as model organism due to its close relation to bacteria causing several pathogens such as tuberculosis, diphtheria and leprosy.

C. glutamicum, like *Mycobacterium tuberculosis*, has a distinct cell wall which is composed of a peptidoglycan layer (murein) with covalently bound polysaccharide layers that are capped with mycolic acids. In addition, both organisms have a polar cell wall synthesis machinery which is spatially regulated by DivIVA (Wag31 in *M. tuberculosis*). The present study shows that DivIVA regulates cell wall synthesis upon direct interaction with the lipid II flippase RodA. RodA determines morphology and growth in *C. glutamicum* and is localized to the poles and septa. The absence of *rodA* results in growth defects and cell shape alterations as well as altered lipid II proliferation of the poles (polar cell growth is sustained). DivIVA is furthermore involved in chromosome segregation upon direct interaction with the partitioning ParB protein, which binds to *parS* sites on the chromosome, thus tethering the replicated nucleoids to the cell poles. Interactions of DivIVA with ParB and RodA were identified in a synthetic *in vivo* protein-protein interaction assay where fluorescently labeled proteins of interest are expressed in *E. coli* cells and interaction is analyzed microscopically. A decisive improvement of this assay is the application of FRET, which is more sensitive and allows quantification of interaction. In order to test whether ParB and RodA compete for the same interaction site in DivIVA, we mapped interaction sites of both proteins. It turned out that ParB binds to a middle region of DivIVA, whereas RodA binds to the N-terminal domain of DivIVA where one lysine residue is essential for interaction.

To fight bacterial infections, that cause thousands of casualties each year, it is mandatory to understand mechanisms in cellular processes, such as cell division and growth, to find new targets for antibiotic intervention. Unfortunately, bacteria are able to develop resistances against many antibiotics. The mycolic acid or arabinan layer and synthesis machinery are good candidates for new antibiotics. Amongst others, two of them have emerged as useful drugs against *M. tuberculosis*, ethambutol (EMB) and BTZ043. In this study, we investigated the modes of action and antibiotic susceptibility of *C. glutamicum a*fter EMB and BTZ043 treatment. We found that both antibiotics, which target the arabinan synthesis pathway, affect exclusively polar elongation growth, as demonstrated in different staining assays. Interestingly, only 10% of the cells were killed and cells in stationary phase were not affected by EMB or BTZ043. Moreover, we used a chromosomal DivIVA-mCherry fusion and found that DivIVA protein level is drastically increased. The cells show asymmetric recovery after

treatment, in which one daughter cell acquires the excess DivIVA whereas the other daughter cell exhibits normal cell growth.

ZUSAMMENFASSUNG

Corynebacterium glutamicum ist ein Gram-positives Bodenbakterium mit großer industrieller Bedeutung für die Herstellung von Aminosäuren im Tonnenmaßstab. Des Weiteren bekommt es zunehmende Bedeutung für die medizinische Forschung, wo es aufgrund seiner engen Verwandtschaft zu den pathogenen Erregern von Tuberkulose, Diphtherie und Lepra als idealer Modellorganismus dient.

Besonders die Zellwand von *C. glutamicum* hat große Ähnlichkeit zu der vieler pathogener Vertreter wie *Mykobakterium tuberculosis*. Sie besteht aus einer Peptidoglycan-Schicht (Murein), an der über weitere Polysaccharid-Schichten die charakteristischen Mycolsäuren gebunden sind. Darüber hinaus besitzen beide Organismen eine polare Zellwandsynthese, die von DivIVA (Wag31 in *M. tuberculosis*) räumlich reguliert wird. Die Rolle von DivIVA am Zellwachstum wurde vor Jahren erstmals beschrieben, jedoch war seine exakte Funktion bis zuletzt unbekannt. In dieser Studie wird erstmals die Funktion von DivIVA am polaren Zellwachstum durch Interaktion mit der Lipid II-Flippase RodA gezeigt. RodA beeinflusst die Morphologie und das Wachstum von *C. glutamicum* und wird von DivIVA an die Zellpole lokalisiert. Deletion von *rodA* resultiert in reduziertem Wachstum und veränderter Morphologie, sowie einer alternativen Lipid II Versorgung der Zellpole, da das polare Zellwachstum erhalten bleibt. DivIVA ist darüber hinaus an der Chromosomensegregation beteiligt, wo es direkt mit ParB interagiert, das über *parS*-Seiten an die replizierten Chromosomen bindet um sie an die Zellpole zu fixieren. Die Interaktionen zwischen DivIVA und ParB bzw. RodA wurden mit Hilfe eines synthetischen *in vivo* Assays identifiziert, worin die zu untersuchenden Gene an Fluorophore gekoppelt und in *E. coli* Zellen exprimiert werden. Somit lässt sich eine Co-Lokalisation nach individueller und Co-Expression der Fusionsproteine mikroskopisch analysieren. Eine entscheidende Verbesserung dieses Assays ist die Verwendung von FRET, das sensitiver ist und eine Quantifizierung der Interaktion ermöglicht. Um herauszufinden, ob ParB und RodA um die gleiche Bindungsstelle an DivIVA konkurrieren, wurden die Interaktionsdomänen beider Proteine ermittelt. Während ParB an eine mittlere Region in DivIVA bindet, bindet RodA an die N-terminale Domäne von DivIVA, in der ein Lysin-Rest für die Bindung essenziell ist.

Für den Kampf gegen bakterielle Infektionskrankheiten, die jährlich tausende Todesfälle verursachen, ist es dringend notwendig zelluläre Mechanismen, beispielsweise der Zellteilung und des Wachstums, zu entschlüsseln um Targets für neue Antibiotika zu finden. Insbesondere die kontinuierliche Entstehung neue Resistenzen macht diese Aufgabe wichtiger denn je. Die Mykolsäureschicht und ihre Synthese sind vielversprechende Targets, da bisher nur wenige Antibiotika, wie Ethambutol (EMB) oder BTZ043, dagegen existieren.

Wir haben die Wirkungsweise und antibiotische Suszeptibilität von *C. glutamicum* nach EMB und BTZ043 Behandlung untersucht. Beide Antibiotika, die in die Arabinogalactan-Synthese eingreifen, beeinflussen ausschließlich das polare Zellwachstum, wie in mehrerer Färbeassays gezeigt. Lediglich 10% der Zellen wurden getötet. Zellen, die sich in der stationären Phase befanden, wurde nicht beeinflusst. Darüber hinaus zeigte die Verwendung eines Stammes mit chromosomaler DivIVA-mCherry Fusion, dass das DivIVA Protein Level stark erhöht ist. Erholungsexperimente nach Antibiotikazugabe zeigten, dass die Zellen asymmetrisch reagieren, wobei eine Tochterzelle das überschüssige DivIVA übernimmt, während die andere Zelle normales Wachstum erfährt.

LIST OF PUBLICATIONS

1) A synthetic *Escherichia coli* system identifies a conserved origin tethering factor in Actinobacteria

   Donovan C, **Sieger B**, Krämer R, Bramkamp M

   Mol Microbiol. 2012 Apr;84(1):105-16. doi: 10.1111/j.1365-2958.2012.08011.x

   Chapter 2.1

2) The lipid II flippase RodA determines morphology and growth in *Corynebacterium glutamicum*

   Sieger B, Schubert K, Donovan C, Bramkamp M

   Mol Microbiol. 2013 Dec;90(5):966-82. doi: 10.1111/mmi.12411

   Chapter 2.2

3) Interaction sites of DivIVA and RodA from *Corynebacterium glutamicum*

   Sieger B, Bramkamp M

   Front. Microbiol. 2014 Dec;5:738. doi: 10.3389/fmicb.2014.00738

   Chapter 2.3

4) The anti-tuberculosis drugs Ethambutol and BTZ043 selectively block elongation growth in CMN-group bacteria

   Sieger B, Schubert K, Rieblinger A, Böhm K, Sachs N, Meyer F, Wanner G, Bramkamp M

   Unpublished manuscript, 2014

   Chapter 2.4

CONTRIBUTIONS TO PUBLICATIONS

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   Boris Sieger contributed experiments to figure 1B, top blot: Purification of DivIVA and ParB and co-elution of DivIVA and ParB as *in vitro* assay for protein-protein interaction and figure 1D: statistical evaluation of DivIVA distribution in *C. glutamicum* (red) and *E. coli* (green). All other experiments were done by Catriona Donovan. The MS was written by Catriona Donovan and Marc Bramkamp. Reinhard Krämer participated in the discussion about the MS.

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   All plasmids, strains, experiments and figures were done by Boris Sieger. HPLC measurements and data analysis were done by Boris Sieger and Karin Schubert. The MS was written by Boris Sieger and Marc Bramkamp. Catriona Donovan and Karin Schubert participated in the discussion about the MS.

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Unpublished manuscript, 2014

Experiment design, data analysis, plasmid and strain construction, figures and experiments for figure 1C,E,F, 2, 5B, 6, S2, S3 were done by Boris Sieger. Az-D-Ala and DHPE staining experiments (figure 3) were done by Fabian Meyer and Karin Schubert. Electron microscopy images were taken by Gerhard Wanner. All other experiments and measurements were done by Angela Rieblinger, Kati Böhm and Nadja Sachs during their practical course / Bachelor thesis under supervision of Boris Sieger. The MS was written by Boris Sieger and Marc Bramkamp.

Chapter 2.4

Prof. Marc Bramkamp Boris Sieger

1 INTRODUCTION

Understanding cellular processes in bacteria is the fundamental prerequisite for bacterial applications in biotechnology or medicine. The engineering of high efficient production strains as well as the treatment of bacterial pathogens have evolved to extremely important tasks in our society. Being found in the 1950's as natural amino acid producer, *Corynebacterium glutamicum* is today one of the most important organisms in biotechnology. It has been engineered to produce a variety of amino acids, of which L-lysine and L-glutamic acid are the most prominent ones. Apart from that, *C. glutamicum* has gained a great medical interest because of its close relationship to pathogens such as *Corynebacterium diphtheriae*, *Mycobacterium leprae* and *Mycobacterium tuberculosis* that all belong to the suborder *Corynebacterineae* (Figure 1, blue box). *C. glutamicum* is a non-pathogenic Actinobacterium and serves as model organism to investigate cellular processes and to find new targets for antibiotic (AB) intervention against the mentioned pathogens. An important characteristic of the suborder *Corynebacterineae* is the Mycobacteria-like cell wall, which contains a mycolic acid layer (MA) at the outer cell wall surface and provides distinct cellular properties (chapter 1.3).

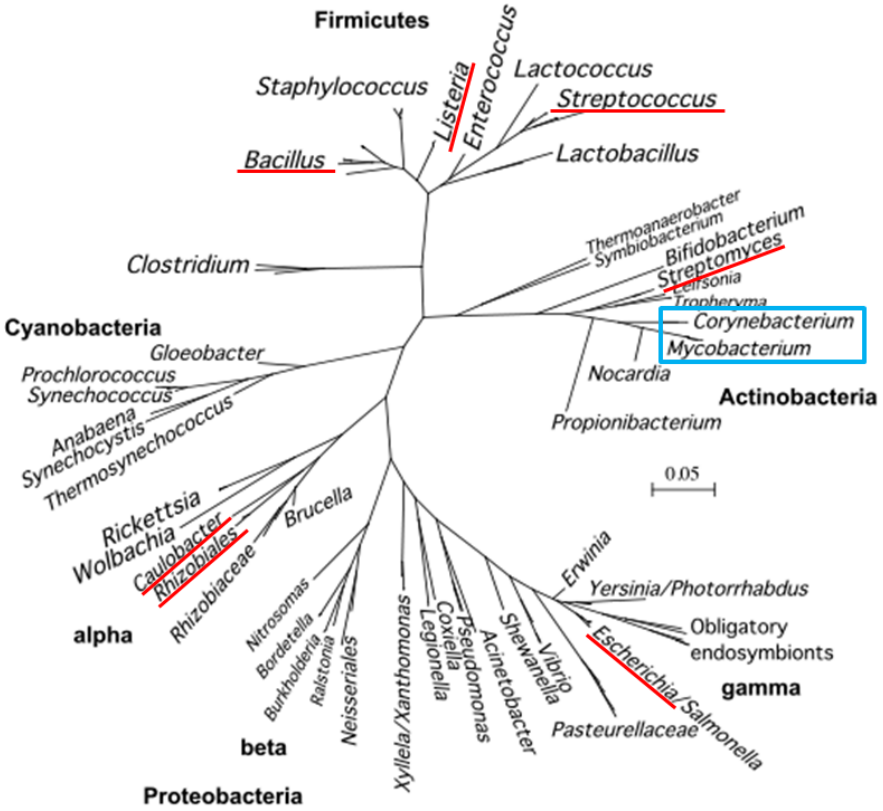

Figure 1: Unrooted phylogenetic tree of bacterial species. The blue box highlights the close relationship of *Corynebacterium* and *Mycobacterium*. Organisms that are underlined in red are mentioned in the text. Modified from (Rocha 2006).

Although a multitude of ABs and targets have been explored in the past decades, bacteria managed (and still manage) to develop multi-resistant strains against established therapies. This impressive evolutional step happens to be alarmingly fast - faster than the progress in AB research. It is thus our duty to further explore yet unknown cellular processes and find new AB targets to fight upcoming multi-resistant strains.

1.1 Cell division and growth in bacteria

The bacterial cell cycle is composed of three fundamental steps: I) cell elongation, II) chromosome replication and segregation and III) septum formation and division.

Within all bacterial phyla, cell elongation happens generally in three distinct modes (Daniel and Errington 2003). To date, these modes are unchallenged, besides minor updates in protein topologies that could be monitored by high-tech microscopy techniques such as PALM and STORM. Actinobacteria, like *Corynebacterium* or *Mycobacterium* elongate from the cell poles, implicating independence of elongation growth from the divisome (Locci and Schaal 1980) (Figure 2A).

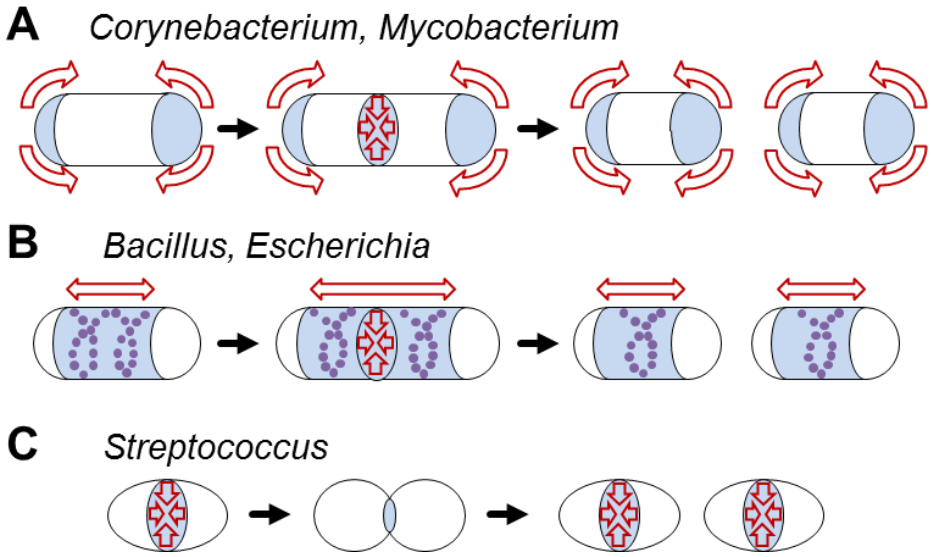

Figure 2: Distinct models of cell wall growth in bacteria, according to (Daniel and Errington 2003). Blue highlights areas of PG synthesis, red arrows show direction of cell wall growth, purple dots describe dynamic MreB filaments. Drawn according to (Daniel and Errington 2003).

Here, cell growth is organized by the polar scaffold protein DivIVA (Wag31 in *M. tuberculosis*), which targets negative membrane curvature at the cell poles and septa (Letek *et al.* 2008b, Kang *et al.* 2008) (chapter 1.2).

Lateral cell growth is mediated by actin-like MreB or Mbl filaments (Jones *et al.* 2001, Vats *et al.* 2009, Dominguez-Escobar *et al.* 2011). MreB interacts with several PBPs and other

conserved membrane proteins (MreC, MreD, RodA, RodZ), thereby determining cell shape (Osborn and Rothfield 2007, Garner *et al.* 2011, Dominguez-Escobar *et al.* 2011). This elongation mode is best analyzed in *E. coli* and *B. subtilis* (Margolin 2009) (Figure 2B).

Cocci have no elongation growth machinery. Their cell wall growth is driven by the division machinery at midcell and cell growth is accompanied with closure of the division site (Figure 2C). After division, the cell poles are completely inert. Consequently, the cells strongly depend on formation of a new division site for cell wall growth (Daniel and Errington 2003).

Independent from the mode of cell growth, all bacteria that possess a cell wall (there are a few exceptions, e.g. mycoplasmas or synthetic L-Forms, chapter 1.3) are equipped with a cell wall growth machinery that is generally composed of 3 major elements: I) Precursor synthesis, followed by II) precursor translocation over the membrane and III) incorporation into the existing cell wall (Figure 3).

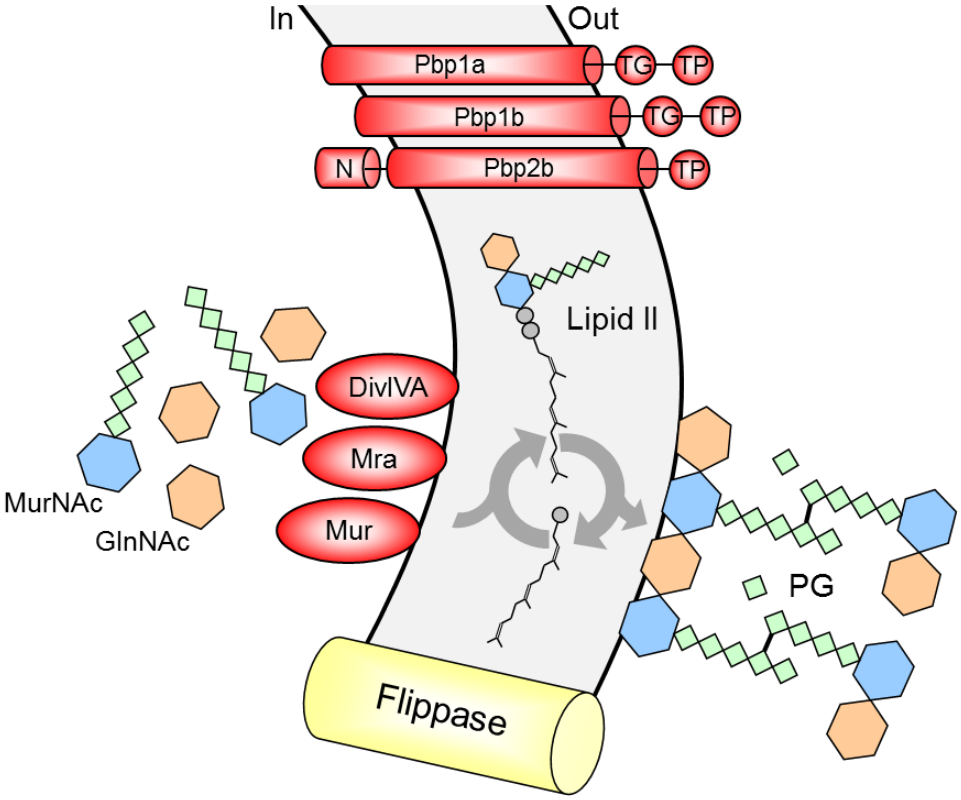

Figure 3: Basic model of PG synthesis in bacteria. PG precursors are synthesized in the cytoplasm and subsequently fused to decaprenyl pyrophosphate, resulting in lipid II. Lipid II is flipped to the outer membrane surface where the disaccharide-pentapeptides are incorporated into the existing cell wall by PBPs. Decaprenyl pyrophosphate is recycled back to the inner membrane site. For a detailed depiction of proteins involved in precursor synthesis see reviews from (Bugg *et al.* 2011, Typas *et al.* 2012, Pinho *et al.* 2013).

Precursor synthesis is proceeded in the cytoplasm, where lipid I and lipid II are synthesized by Mur and Mra proteins, respectively (chapter 1.3 for detailed explanation). Subsequently,

lipid II is translocated by lipid II flippases such as FtsW, RodA or SpoVE, members of the SEDS (shape, elongation, division, sporulation) protein family. The best described representative of this protein class is FtsW. It was shown by reconstitution experiments and FRET analysis, how FtsW mediates lipid II translocation over membranes *in vitro* (Mohammadi *et al.* 2011). RodA and FtsW from *E. coli* share 31.9% identity of a 320 AA overlap and *E. coli* FtsW and *B. subtilis* SpoVE share 39.8% identity of a 352 AA overlap (Ikeda *et al.* 1989). The immense degree of identity between FtsW, RodA and SpoVE leads to the idea that all three proteins fulfill the same function. *C. glutamicum* has homologues of both SEDS proteins, FtsW and RodA. While FtsW, gene product of a member of the *fts* operon and thus part of the divisome, acts in septal PG synthesis during division, RodA is involved in polar PG synthesis during cell elongation (Valbuena *et al.* 2007). SpoVE is involved in lipid II translocation during sporulation (Ikeda *et al.* 1989) and consequently not present in the non-sporulating organisms *E. coli* or *C. glutamicum*.

During cell elongation, the chromosomes start to replicate beginning at the origin of replication towards the terminus region. Meanwhile, the replicated chromosomes are segregated and anchored to the cell poles. In *C. glutamicum*, chromosome partitioning and anchoring is fulfilled by the ParAB system, where ParA, a walker-type ATPase, drives the segregation process and ParB anchors the chromosomes via *parS* sites to the cell poles (Donovan *et al.* 2010).

In *E. coli* or *B. subtilis*, division site selection is mediated by systems such as Min, Noc and SlmA to determine midcell with high precision (de Boer *et al.* 1989, Wu and Errington 2004, Bernhardt and de Boer 2005). Interestingly, *C. glutamicum* lacks all three mentioned division site determining machineries. As a consequence, division site is not precisely at midcell, like it is in *B. subtilis* or *E. coli*. Instead it appears at a random position in between the segregated chromosomes. Encountered also in WT cells, especially mutants defective of chromosome segregation show aberrant placement of division site with septa that often assemble and constrict over the nucleoids (Donovan *et al.* 2010, Donovan *et al.* 2013). The division process is initiated by early stage components of the divisome at division site. FtsZ, for instance, appears together with several regulators (SsgB, ZipA, ZapA, EzrA) to assemble the Z-ring in *E. coli* and *B. subtilis* (Lutkenhaus *et al.* 1980, Bi and Lutkenhaus 1991, Scheffers 2005, Son and Lee 2013, Hale and de Boer 1997, Gueiros-Filho and Losick 2002, Levin *et al.* 1992). Afterwards, late stage division proteins such as PBP3/FtsI and FtsW follow towards the division site to fulfill septum closure and cell wall growth during division, as shown for *M. tuberculosis* (Datta *et al.* 2002, Datta *et al.* 2006). Finally, the division site is completed by proteins like DivIVA, which recognizes newly formed negative membrane curvature at the septum (Lenarcic *et al.* 2009).

1.2 The versatile polar determining scaffold protein DivIVA

DivIVA is one of the most important cell division proteins and has been extensively discussed in the past decades. It is widely conserved within Gram positive bacteria and has the ability to localize to negative membrane curvature as observed at the cell poles and septa (Lenarcic *et al.* 2009). Consequently, it is considered as polar determinant and organizer for a large number of cellular processes. DivIVA from *B. subtilis* ($DivIVA_{Bsu}$) was first described in the late 1990's and is the best characterized homologue to date (Edwards and Errington 1997). Ever since, DivIVA homologues have been identified and described in several bacterial phyla. DivIVA is composed of a highly conserved short N-terminal domain, followed by two coiled coil domains CC1 and CC2 (Letek *et al.* 2008). It was shown by crystal structure of the N-terminal domain of $DivIVA_{Bsu}$, that an exposed hydrophobic AA residue (phenylalanine) is responsible for polar and septal membrane attachment (Oliva *et al.* 2010). Moreover, DivIVA forms oligomers, mediated by the coiled coil domains, that provide scaffold formation (Stahlberg *et al.* 2004). The oligomers have been identified as 10-12-mers by ultracentrifugation and gel permeation techniques (Muchova *et al.* 2002). DivIVA from *B. subtilis* functions as division site marker where it forms a ring like structure (Edwards and Errington 1997). Furthermore, it serves as spatial regulator of the Min system, where it bridges the FtsZ inhibitor MinCD via MinJ to the cell poles to prevent cell division apart from midcell (Bi and Lutkenhaus 1993, Marston *et al.* 1998, Bramkamp *et al.* 2008, Patrick and Kearns 2008). During cell differentiation, *B. subtilis* DivIVA functions in chromosome segregation during sporulation where it interacts with RacA to tether the nucleoid into the prespore (Thomaides *et al.* 2001, Ben-Yehuda *et al.* 2003). During asymmetric division before sporulation, DivIVA localizes to the polar septum where it is required for activation of σ-factor F (Eswaramoorthy *et al.* 2014). Since both events require the presence of the sporulation protein SpoIIE it could be shown with high resolution microscopy that both proteins interact and co-localize at the site of the polar septum which faces the smaller (spore) compartment. Moreover, *B. subtilis* DivIVA mediates polar and septal localization of ComN upon direct interaction (dos Santos *et al.* 2012). ComN is a posttranscriptional factor of the competence operon *comE*. The absence of DivIVA (Δ*divIVA*) leads to delocalization of ComN and thus to reduced competence efficiency, demonstrating how DivIVA is directly involved in DNA uptake in *B. subtilis*. Although it had been considered as independent polar determinant, it could be shown very recently that DivIVA targeting in *B. subtilis* depends on SecA (Halbedel *et al.* 2014). SecA is an ATPase for protein secretion and interacts directly with DivIVA, as shown in pull down experiments. In the absence of SecA, DivIVA is delocalized, likely due to disadvantages in membrane binding. However, since SecA is involved in protein secretion and membrane insertion, its role in DivIVA localization is likely

indirect (Halbedel *et al.* 2014). In *Listeria monocytogenes*, DivIVA is involved in secretion of autolysins, such as p60 and MurA (Halbedel *et al.* 2012). A Δ*divIVA* mutant of this organism showed a chaining phenotype, but had no division defects like it was observed in Δ*divIVA* mutants of other organisms (Fadda *et al.* 2003, Letek *et al.* 2008b, Kang *et al.* 2008). This chaining phenotype is most likely due to reduced extracellular levels of the mentioned autolysins. A similar phenotype was observed for the Δ*secA2* mutant with malfunctions in the SecA2 secretion machinery. Moreover, in this context, the effect of DivIVA on swarming motility, biofilm formation, invasiveness and cell-to-cell spread in cell culture models could be shown, suggesting the importance of DivIVA in *L. monocytogenes* (Halbedel *et al.* 2012). DivIVA's function in polar cell growth was first described for *Streptomyces coelicolor* (Flardh 2003), and since then also observed in several other Actinomycetales species (Kang *et al.* 2008, Letek *et al.* 2008b), however, the precise role was not clarified, yet. In *S. coelicolor,* DivIVA is part of a tip organizing center, where it interacts with the coiled coil protein Scy (Streptomyces cytoskeletal element) and intermediate filament like protein FilP (Holmes *et al.* 2013, Fuchino *et al.* 2013). The absence and overproduction of Scy results in altered morphology and branching with mislocalization of proteins, suggesting a spatio-regulatory role of Scy. FilP assembles into a network structure to provide cellular rigidity and elasticity during polar elongation. Both proteins are spatially regulated by DivIVA and the resulting tip-associated DivIVA complex is considered as a platform for several more spatio-dependent processes, that are summarized and named as the polarisome or TIPOC (Flardh *et al.* 2012, Fuchino *et al.* 2013). In *M. tuberculosis*, polar cell growth and morphology are determined by the DivIVA homologue Wag31, which is essential for cell growth (Kang *et al.* 2008). Here, Wag31 interacts with AccA3 and is involved in lipid permeability, whereas overexpression of AccA3 results in a decrease of such (Xu *et al.* 2014). Consequently, Wag31 plays a role in maintaining drug resistance and lipid permeability of the cell wall. In *C. glutamicum*, DivIVA governs spatial regulation of PG synthesis through polar recruitment of the PBPs upon weak interaction between PBP1a and DivIVA, as shown in a bacterial-two-hybrid assay (Valbuena *et al.* 2007).

The polymerization of DivIVA is nucleotide independent, meaning that scaffold formation and polar localization does not require energy. However, for proper functioning and regulation of growth, it needs to be phosphorylated by STPKs. In *Streptococcus pneumoniae*, a triad composed of DivIVA, GpsB (a DivIVA paralog) and StkP (a protein kinase) has been identified to regulate PG synthesis, whereas GpsB is required for StkP localization, which in turn phosphorylates DivIVA (Fleurie *et al.* 2014). Interestingly, they found that cell elongation is promoted by non-phosphorylated DivIVA, whereas phosphorylation abolishes elongation growth and stimulates septal PG synthesis and division (Fleurie *et al.* 2012). In *M.*

tuberculosis, phosphorylation of Wag31 affects not only the enzymatic activity of polar PG synthesis but also protein-protein interactions (Jani *et al.* 2010). *C. glutamicum* harbours 4 STPKs, namely PknA, PknB, PknG and PknL, whereas only two of them, PknA and B are essential (Fiuza *et al.* 2008). Moreover, the protein kinases A, B and L were found to phosphorylate FtsZ (Schultz *et al.* 2009). Phosphorylation of *C. glutamicum* DivIVA has not been demonstrated, yet.

Albeit the versatility of DivIVA, it is highly restricted to Gram positive organisms. Nevertheless, polar determinants are a general prerequisite in spatial control of the cell cycle. Gram negative bacteria have therefore other proteins to fulfil these functions that are partly comparable with those of DivIVA. In *E. coli,* for instance, the Min system for division site selection is replenished with the topological factor MinE which tethers MinCD to the cell poles (de Boer *et al.* 1989). *Caulobacter crescentus*, an oligotrophic fresh water bacterium with asymmetric cell division has PopZ as polar determinant which is involved in chromosome partitioning (Bowman *et al.* 2008, Laloux and Jacobs-Wagner 2013). Neither MinE nor PopZ have structural similarities compared to DivIVA, however, like DivIVA, PopZ requires multimerization to form a matrix for polar localization (Laloux and Jacobs-Wagner 2013). Regarding the essentiality of DivIVA homologues, those in Actinobacteria seem to be the most important ones. *C. glutamicum divIVA* is essential for viability and is required for polar cell growth (Letek *et al.* 2008b). Likewise its homologues from other organisms, $DivIVA_{Cgl}$ consists of three distinct domains, a short N-terminal domain and two coiled-coil domains (Letek *et al.* 2009). However, the protein has a large central insertion, which gives hints to further functions (Letek *et al.* 2009). It could for instance be a good candidate for polar tethering of the previously described ParAB system during chromosome segregation (Donovan *et al.* 2010).

1.3 The bacterial cell wall

Bacteria are generally classified into Gram positives and Gram negatives, according to the Danish bacteriologist Hans Christian Gram. Experimental basis for this classification, that was first described in 1886, was a staining procedure which stained Gram positive cells dark purple and Gram negatives (upon counter staining) red. Later it was found by electron microscopy analysis, that this difference in coloring is owing to the structure of the cell wall. Gram positive bacteria have a thick PG layer (20-80 nm), whereas Gram negative cell walls are much thinner (6-8 nm, Figure 4).

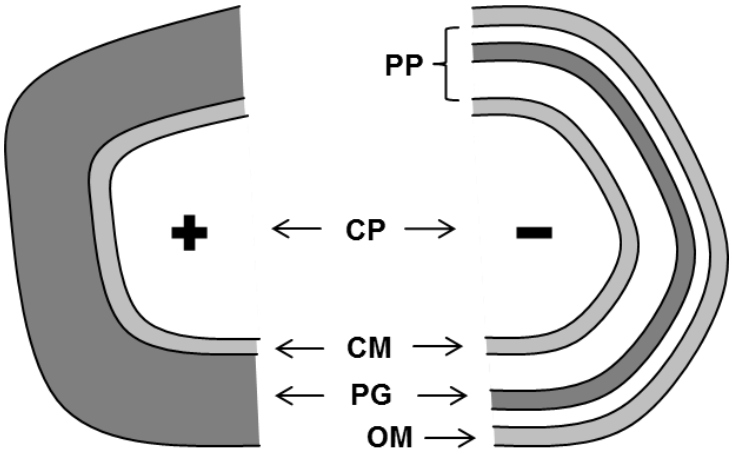

Figure 4: Cell wall scheme of Gram positive (left) and Gram negative (right) bacteria. CM = cell membrane, CP = cytoplasm, OM = outer membrane, PG = peptidoglycan, PP = periplasm.

Only a few organisms are not distinguishable with this method, as they show false or mixed results, and are therefore classified as Gram variable. Examples are PG free Mollicutes, Gram positive *Deinococcus* that stain according to their Gram negative-like cell wall or *Bacillus*, which show mixed staining patterns upon PG degradation (Thompson and Murray 1981, Miyata and Ogaki 2006). Bacterial cell walls are flexible molecular networks that protect the cells from outer environmental influences, like mechanical erosion in soil, and from their internal turgor pressure, caused by osmotic flow of water into the cell. They are generally made of PG (Murein), which is composed of a polysaccharide (PS) backbone consisting of alternating β-(1,4) linked N-acetyl glucosamine (GlnNAc) and N-acetyl muramic acid (MurNAc) molecules (Schleifer and Kandler 1972). Single PG strands are cross-linked via short peptide bridges between MurNAc elements, thus forming a mesh-like layer (called sacculus) that gives the cell wall its structural integrity and strength. These pentapeptide cross-links differ between organisms, no matter if Gram positive or negative, and some even lack PG cross-links. The pentapeptide cross-links are usually made of five AAs or AA derivatives, in particular L-Alanine, D-glutamate, *meso*-diaminopimelic acid (DAP, *E. coli, C. glutamicum, B. subtilis*) / L-lysine (*S. aureus*) and two D-alanine. The choice of D-AAs is believed to help protect the cell wall from protease attacks, since D-AAs do not occur in proteins. Like all components of the cell wall, the cross-links vary between all organisms and many exceptions have been described (Schleifer and Kandler 1972). The PG precursor molecules are synthesized in the cytoplasm. The reaction cascade has 12 steps, beginning with fructose-6-phosphate, an intermediate from glycolysis (van Heijenoort 2007). In the last step MurNAc-(pentapeptide)-pyrophosphate (lipid I) is fused to GlnNAc by the glycosyltransferase MurG, yielding lipid II. The lipid II is then flipped to the outer membrane surface by lipid II flippases, as mentioned. After translocation, PBPs with transglycosylation

activity insert the subunits into existing glycan strands and the cross-linking is fulfilled by PBPs with transpeptidation activity. On top of the PG, some Gram positive species have additional PS layers that contribute to the thickness of the cell wall (**Figure 5**).

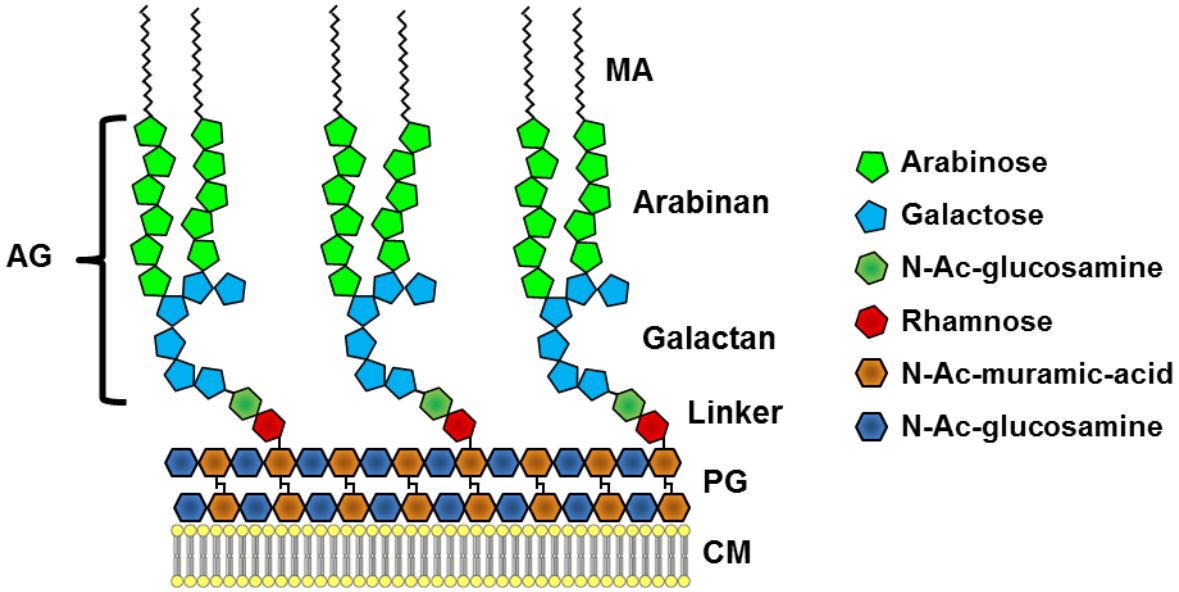

Figure 5: Basic model of the Mycobacteria cell wall. Galactan chains are esterified with arabinan chains to form the AG layer, which is connected to the PG via a short linker. The Arabinan chains are capped with mycolic acids. Drawn according to (Bhamidi *et al.* 2008).

The suborder of *Corynebacterineae*, including *C. glutamicum* and *M. tuberculosis*, has a distinctive AG layer which is covalently bound to PG via short linker molecules and is made of D-galactose and arabinose strands that are attached at three branch points. The arabinose strands are 30 monomers long and capped with mycolic acids (Bhamidi *et al.* 2008). This mycolic acid layer has a characteristic length of ~90 C-atoms in *Mycobacteria*. *Corynebacteria* also possess mycolic acids, however their residues are much shorter (~40 C-atoms). This particular fact makes *C. glutamicum* an interesting model organism to study mycolic acid layer formation in order to find new targets for AB intervention.

The cell wall is essential for most bacteria to survive, although it is possible to produce cell wall lacking L-forms of several bacteria and cultivate them in the laboratory (Tulasne 1949, Mercier *et al.* 2014). Very few bacteria lack cell walls, such as *Mycoplasma*, most of which are pathogenic and to date one of the smallest living cells discovered.

1.4 Cell wall antibiotics

A milestone in the treatment of bacterial infections was established by Alexander Fleming in 1928 when he found that fungi of the genus *Penicillium* excrete a substance that kills certain bacteria. Later, he found that this substance, which he named penicillin, kills specifically

Gram positive bacteria, whereas Gram negative *Salmonella*, for instance, appeared to be resistant. Penicillin and its derivatives are structurally based on a beta-lactam ring and are therefore summarized as beta-lactam ABs. The beta-lactam ring binds covalently and irreversible to D-alanine transpeptidase enzymes, thereby inhibiting cross-linking of the glycan strands in Gram positive bacteria, leading to perforation and burst. Due to the fact that these transpeptidase enzymes were barely characterized at that time, they were named according to this reaction – penicillin binding proteins (PBPs). Since then several derivatives of the same class of antibiotics were developed with higher stability, effectiveness and production yield in biotechnological production. Beta-lactam ABs are classified as bactericidal, as they act by inhibiting reproduction. Well established ABs of this class are penicillin and cephalosporin (including several derivatives). The drawback, however, of these ABs lies in their structural basis, the beta-lactam ring. Resistant bacteria harbor beta-lactamases that break and thus inactivate the beta-lactam ring. In addition, due to the widespread and sometimes inappropriate use of ABs, bacteria developed further resistances against many established ABs. It is possible to counteract resistance for example by adding inhibitors for beta-lactamases. Mixtures of such, often expanded by further ABs, are summarized as broad-spectrum ABs. To counteract the rapid development in evolution, a variety of new AB had to be developed, targeting in the meantime most cellular elements or processes (e.g. ribosomes → protein synthesis, cell wall → cell growth, cell membrane → transport, DNA → chromosome replication).

A well-studied and established AB target is the bacterial cell wall. One reason is the huge structural variety and composition between different organisms, which results in differences in AB susceptibility between bacteria (chapter 1.2). Furthermore, it has the advantage, that ABs do not require uptake into the cell and thus have no barrier that needs to be passed. The targets of the cell wall extend from its synthesis machinery (PBPs), over precursor molecules (lipid II) (Breukink and de Kruijff 2006) to all different layers that are present, such as PG, AG, MA, etc.

One of the most problematic diseases caused by bacterial infections is TB, which is still in focus of AB research since it was not yet possible to find appropriate antisera. Although approx. one third of the world population is infected, only 5-10 % of those develop an active version of such, which causes severe inflammations of lungs accompanied with chest pains. Strikingly, latent pathogens are able to survive for years, and therapies are long-drawn and expensive. In 2013, TB caused 1.1 million casualties worldwide (WHO).

The mycobacterial cell wall not only provides cellular robustness but is also essential for growth, virulence and survival, thus makes it an interesting target for new ABs (Portevin *et al.*

2004). One promising drug that has been identified recently is BTZ043, the most effective one of the benzothiazinone compound family (Makarov *et al.* 2009). BTZ043 targets the essential DprE1 catalytic protein that is involved in isomerization of the cell wall precursor molecule 1-decaprenylphosphoryl ribose (DPR) to the oxoderivative DPX (Wolucka 2008, Crellin *et al.* 2011) (Figure 6).

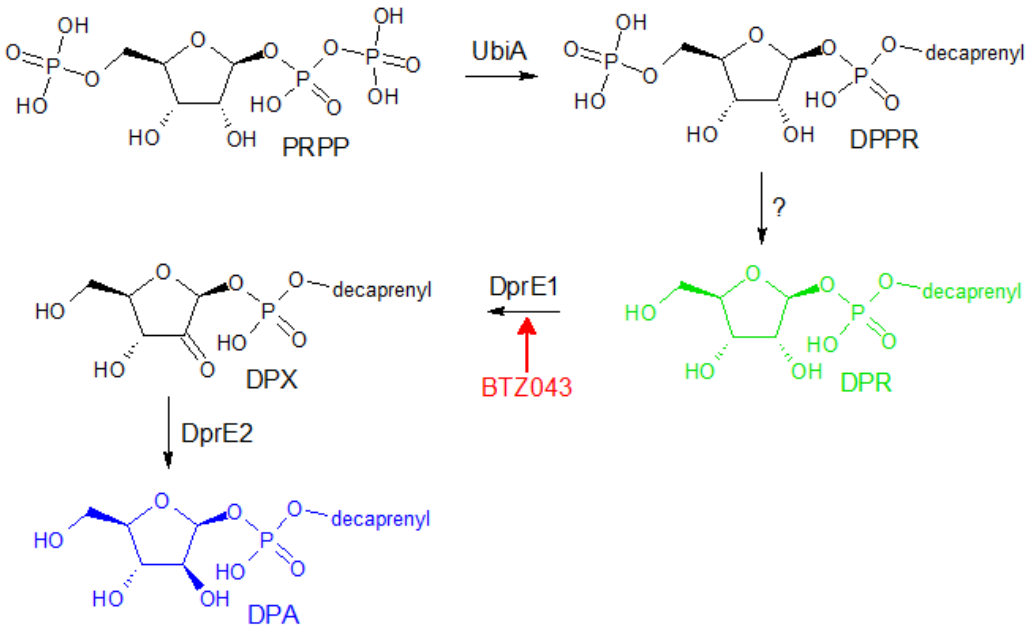

Figure 6: Synthesis pathway of DPA, the precursor for arabinan synthesis, and site of action of BTZ043. At first, 5-phosphoribose-1-pyrophosphate (PRPP) is fused to the decaprenyl chain which serves as membrane carrier (similar to lipid II) by UbiA resulting in 1-decaprenylphosphoryl ribose 5-phosphate (DPPR). DPPR is then dephosphorylated by an unknown protein resulting in DPR. Finally, DPR isomerization to DPA via DPX is fulfilled by DprE1 and 2. DprE1 is the target for BTZ043. Drawn according to (Sassetti *et al.* 2001, Mikusova *et al.* 2005, Wolucka 2008).

DPX is subsequently further proceeded by DprE2 to 1-decaprenylphosphoryl arabinose (DPA), the precursor for arabinan and LAM synthesis. Structural basis for BTZ043-mediated killing of *M. tuberculosis* is a covalent binding of BTZ043 to an active-site cysteine in DprE1 and contact to a neighboring catalytic lysine, as shown by crystal structure of the DprE1-BTZ043 complex (Neres *et al.* 2012). This explains the high potency and MIC of only 1 ng/ml against *M. tuberculosis*. BTZ043 is currently in late stages of preclinical development and will likely be applied in the near future (Lienhardt *et al.* 2012). One established anti-tubercular drug is ethambutol (EMB) which targets the EmbABC arabinosyltransferases for arabinan and LAM biosynthesis (Escuyer *et al.* 2001, Alderwick *et al.* 2005) (Figure 7).

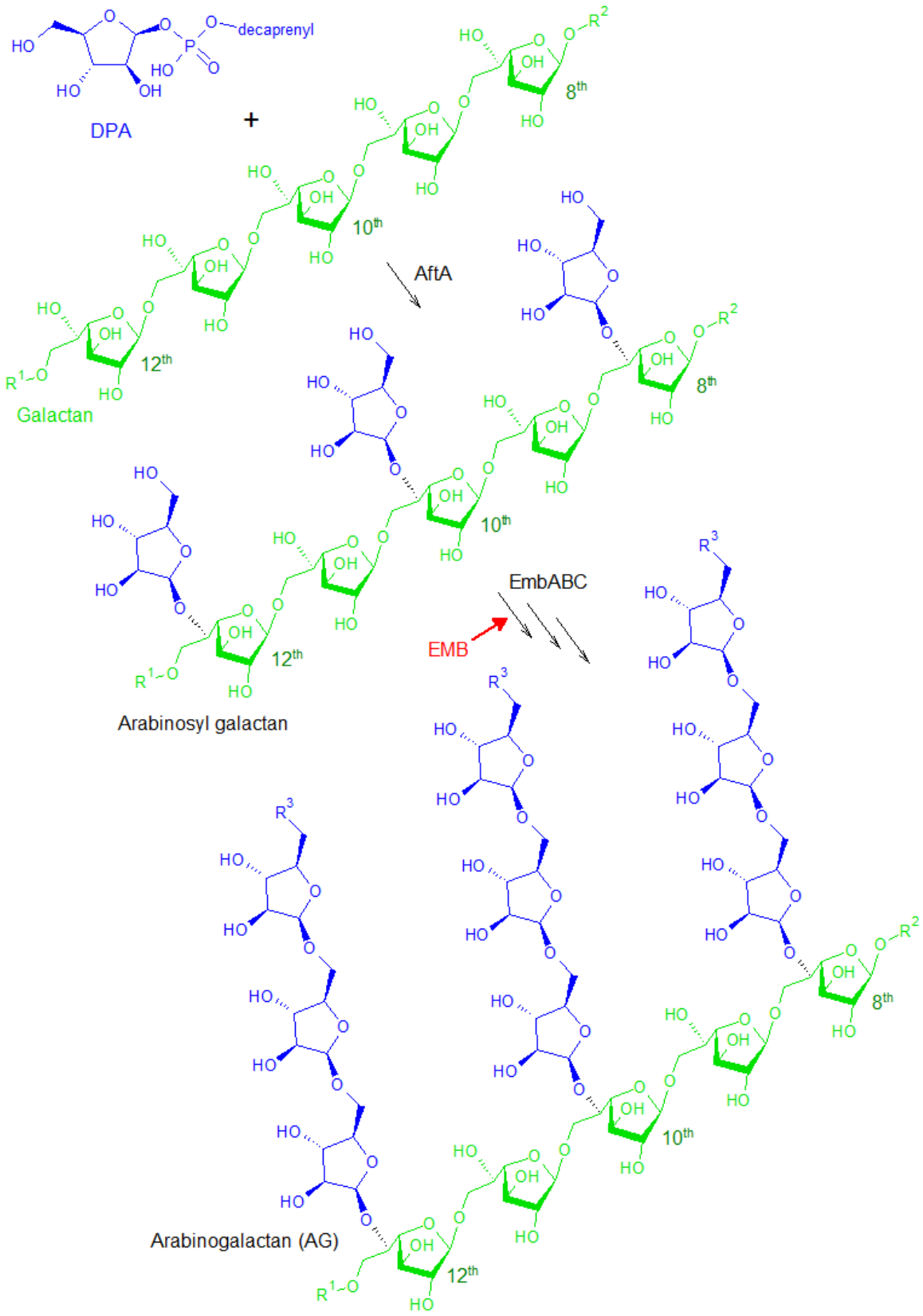

Figure 7: Arabinan synthesis cascade and site of action of EMB. DPA is connected to the galactan chain at galactose 8, 10 and 12 by the arabinosyltransferase AftA, resulting in arabinosyl galactan. Processive addition of arabinose subunits derived from DPA is catalyzed by the arabinosyltransferases EmbAB (*M. tuberculosis*) or EmbC (*C. glutamicum*). Drawn according to (Escuyer *et al.* 2001, Alderwick *et al.* 2005, Alderwick *et al.* 2006).

EMB has been first described in the 1960's and is widely used in TB therapy. In the meantime, however, several resistances occurred, especially due to mutations in the *emb* genes (Cui *et al.* 2014). This is just one example to demonstrate the urgency for the development for new ABs.

1.5 Aim of this work

In *C. glutamicum*, chromosome partitioning is mediated by the ParAB proteins, which have been characterized and described previously in our lab (Donovan *et al.* 2010). ParA is a walker-type ATPase and ParB a DNA binding protein (chapter 1.1). However, for proper tethering of the chromosomes to the cell poles, more components are required. It could be shown that the *oriC-parS*-ParB nucleoprotein complex is located at the poles in *C. glutamicum* (Donovan et al. 2010). A good candidate for polar tethering is DivIVA, which localizes to the poles in *C. glutamicum* (Letek *et al.* 2009). For *B. subtilis* it has been shown that the conserved DivIVA protein is involved in chromosome tethering during sporulation (Thomaides *et al.* 2001). The study that is presented in chapter 2.1 aims to clarify the mechanisms and regulation of chromosome segregation and tethering in *C. glutamicum* and other actinobacterial species.

Cell wall growth has been extensively investigated in many species. The components of the growth machineries can be summarized in three major groups: I) PBPs that incorporate PG precursors into the cell wall (e.g. PBP1, PBP2, PBP3/FtsI), II) lipid II flippases which mediate precursor translocation over the membrane (e.g. RodA, FtsW) and III) several cytosolic proteins for precursor synthesis (e.g. MurG, MraY). *C. glutamicum* exhibits polar elongation growth, a common feature of Actinobacteria, including several pathogens. Consequently, these species are equipped with distinct proteins that form the polar elongation complex, in addition to the septal cell wall growth machinery during division. Although many proteins for polar elongation growth have been identified, it remains a mystery how they are recruited to the cell poles. A role of DivIVA in polar cell growth has been suggested but has not been characterized, yet (Letek *et al.* 2008b). The study presented in chapter 2.2 aims to characterize the lipid II flippase RodA, a member of the SEDS protein family, who's role and essentiality in morphology and growth has not been investigated. Moreover, this study presents how polar elongation complex is recruited to the cell poles.

The conserved cell division protein DivIVA has been identified in several actinobacterial species and many interaction partners have been found. Moreover, the versatile functions of DivIVA have been described (chapter 1.2). It is likely involved in chromosome tethering and polar cell growth and possibly the connection hub between both processes. The study

presented in chapter 2.3 aims to analyze the specificity of the interaction of DivIVA with RodA and to find evidence that DivIVA is required for spatial cell cycle regulation. Approaching the validation of protein-protein interaction candidates, this study aims to establish a new protein-protein interaction assay. Fluorescence microscopy and FRET are promising methods to visualize and quantify protein-protein interaction *in vivo*. Thereby, a heterologous *E. coli* system allows observation of direct physical interaction. Co-elution assays serve as *in vitro* studies.

Altogether, this study aims to give new insights into the interesting and challenging field of spatial cell cycle regulation. One protein in focus is DivIVA, which localizes to the cell poles and septa and is thus a good candidate as spatial regulator and thus connection hub between polar cell growth, chromosome segregation and cell division in *C. glutamicum*.

The close relationship to several pathogens makes *C. glutamicum* an interesting model organisms to study medical aspects such as antibiotic susceptibility and stress response of Actinobacteria. Especially the mycobacteria-like cell wall allows investigation of such, which is problematic in Mycobacteria where it is essential for viability. The study in chapter 2.4 aims to present latest results on AB susceptibility, stress response and cell recovery after (incomplete) AB therapy.

2 RESULTS / PUBLICATIONS

2.1 A synthetic *Escherichia coli* system identifies a conserved origin tethering factor in Actinobacteria

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Abstract

In eukaryotic and prokaryotic cells the establishment and maintenance of cell polarity is essential for numerous biological processes. In some bacterial species, the chromosome origins have been identified as molecular markers of cell polarity and polar chromosome anchoring factors have been identified, for example in *Caulobacter crescentus*. Although speculated, polar chromosome tethering factors have not been identified for Actinobacteria, to date. Here, using a minimal synthetic *Escherichia coli* system, biochemical and *in vivo* experiments, we provide evidence that *Corynebacterium glutamicum* cells tether the chromosome origins at the cell poles through direct physical interactions between the ParB–*parS* chromosomal centromere and the apical growth determinant DivIVA. The interaction between ParB and DivIVA proteins was also shown for other members of the Actinobacteria phylum, including *Mycobacterium tuberculosis* and *Streptomyces coelicolor*.

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Link to publication: http://www.ncbi.nlm.nih.gov/pubmed/22340668

Supplementary material and methods for chapter 2.1

A synthetic *Escherichia coli* system identifies a conserved origin tethering factor in Actinobacteria

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Link to supplementary material:

http://onlinelibrary.wiley.com/store/10.1111/j.1365-2958.2012.08011.x/asset/supinfo/MMI_8011_sm_FigureS1-6_TableS1-3.pdf?v=1&s=c9a859aeda89e1786897bc813a8a52e5a503f66c

2.2 The lipid II flippase RodA determines morphology and growth in *Corynebacterium glutamicum*

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Accepted 21 September, 2013

Abstract

Lipid II flippases play an essential role in cell growth and the maintenance of cell shape in many rod-shaped bacteria. The putative lipid II flippase RodA is an integral membrane protein and member of the SEDS (shape, elongation, division and sporulation) protein family. In contrast to its homologues in *Escherichia coli* and *Bacillus subtilis* little is known about the role of RodA in Actinobacteria. In this study, we describe the localization and function of RodA in *Corynebacterium glutamicum*, a rod-shaped, apically growing Actinobacterium. RodA-GFP localizes exclusively at the cell poles. Surprisingly, time-lapse microscopy revealed that apical cell growth is sustained in a *rodA* deletion strain. However, growth rates and antibiotic susceptibility are altered. In the absence of RodA, it appears that apical growth is driven by lateral diffusion of lipid II that is likely flipped by the septal flippase, FtsW. Furthermore, we applied a previously described synthetic *in vivo* system in combination with FRET to identify an interaction between *C. glutamicum* RodA and the polar growth organizing protein DivIVA.

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Supplementary material and methods for chapter 2.2

The lipid II flippase RodA determines morphology and growth in *Corynebacterium glutamicum*

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Link to supplementary material:

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2.3 Interaction sites of DivIVA and RodA from *Corynebacterium glutamicum*

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Accepted 5 December, 2014

Abstract

Elongation growth in Actinobacteria is localized at the cell poles. This is in contrast to many classical model organisms where insertion of new cell wall material is localized around the lateral site. We previously described a role of RodA from *Corynebacterium glutamicum* in apical cell growth and morphogenesis. Deletion of *rodA* had drastic effects on morphology and growth, likely a result from misregulation of penicillin-binding proteins and cell wall precursor delivery. We identified the interaction of RodA with the polar scaffold protein DivIVA, thus explaining subcellular localization of RodA to the cell poles. In this study, we describe this interaction in detail and map the interaction sites of DivIVA and RodA. A single amino acid residue in the N-terminal domain of DivIVA was found to be crucial for the interaction with RodA. The interaction site of RodA was mapped to its cytoplasmic, C-terminal domain, in a region encompassing the last 10 AAs. Deletion of these 10 amino acids significantly decreased the interaction efficiency with DivIVA. Our results corroborate the interaction of DivIVA and RodA, underscoring the important role of DivIVA as a spatial organizer of the elongation machinery in *Corynebacterineae*.

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Supplementary material and methods for chapter 2.3

Interaction sites of DivIVA and RodA from *Corynebacterium glutamicum*

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Link to supplementary material:

http://journal.frontiersin.org/Article/DownloadFile/142499/octet-stream/Data%20Sheet%201.DOCX/322

2.4 The anti-tuberculosis drugs Ethambutol and BTZ043 selectively block elongation growth in CMN-group bacteria

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Unpublished MS

Abstract

It is widely known that antibiotic research is of growing importance to combat the development of multi-resistant strains that cause lethal infections, such as tuberculosis (TB), diphtheria and leprosy. The WHO counted 1.1 million deaths upon TB infection excluding HIV in 2013, showing how severe they are and how poor our ability to counteract resistances is. Many members of the CMN-group bacteria (*Mycobacterium*, *Corynebacterium*, *Nocardia*) are notorious pathogens and show a high rate of survival against established antibiotics. A first line antibiotic for TB treatment is ethambutol that is part of broad-spectrum therapies and targets the EmbABC arabinosyltransferases for arabinan synthesis. Another antibiotic that has been described recently is BTZ, which targets the isomerization process of decaprenylphosphoryl ribose to decaprenylphosphoryl arabinofuranose. BTZ043 is highly promising due to its low minimal inhibitory concentration of only 1 ng/ml for Mycobacteria. We investigated the mode of action of EMB and BTZ043 on *Corynebacterium glutamicum*, a non-pathogenic relative of *Mycobacterium tuberculosis*. Both organisms elongate from the cell poles and share a remarkably similar cell wall. In addition, the genes that are involved in cell wall synthesis are likewise homologues. We found that EMB / BTZ043 treated cells were shorter due to a selective block of the mycolic acid and peptidoglycan synthesis at the cell poles. Although polar growth was abolished, septal cell wall synthesis continued, thus likely reasoning the survival of the cells.

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Supplementary material and methods for chapter 2.4

The anti-tuberculosis drugs Ethambutol and BTZ043 selectively block elongation growth in CMN-group bacteria

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Unpublished MS

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3 CONCLUDING DISCUSSION

C. glutamicum is a member of the CMN-group bacteria (also called *Corynebacterineae*) to which many pathogenic species with high survival rates against established ABs belong. Although *C. glutamicum* is non-pathogenic, the structure and synthesis of *C. glutamicum* cell wall components is similar to *Mycobacteria* (Dover *et al.* 2004). The cell wall is composed of a PG sacculus that is connected via short linker sequences to a characteristic AG layer (McNeil *et al.* 1990) (Figure 5). The AG layer is capped with mycolic acids that are essential for viability in *Mycobacterium* (Gande *et al.* 2007, Kolly *et al.* 2014). In addition, the mode of cell wall synthesis of *Corynebacterium* and *Mycobacterium* is similar. During division, septal cell wall growth is fulfilled by Fts proteins, such as the topological marker FtsZ, lipid II flippase FtsW and PBP FtsI. This septal growth machinery is unique in most bacterial species (Bi and Lutkenhaus 1991, Weiss *et al.* 1999, Datta *et al.* 2006). Elongation of rod shaped bacterial cells, such as *E. coli* and *B. subtilis*, is achieved by inserting new PG subunit along the lateral cell axis (Osborn and Rothfield 2007). In *Corynebacterium* and *Mycobacterium* the elongation machinery is located at the poles (Daniel and Errington 2003, Letek *et al.* 2008a). Here, DivIVA acts as topological marker, as it self-localizes to the cell poles where it recognizes negative membrane curvature (Lenarcic *et al.* 2009). Although the involvement of *C. glutamicum* DivIVA in cell growth had been proposed (Letek *et al.* 2008b), the exact role in terms of protein interaction or spatiotemporal regulation was not clarified. We identified two interaction partners of *C. glutamicum* DivIVA: The lipid II flippase RodA and the chromosome partitioning protein ParB, thus characterizing the exact role of DivIVA in polar cell growth and chromosome segregation.

3.1 DivIVA spatially regulates chromosome tethering in *C. glutamicum*

Bacteria employ specialized chromosome segregation systems that have been well described (Jakimowicz *et al.* 2002, Wu and Errington 2003, Ramirez-Arcos *et al.* 2005, Schumacher and Funnell 2005, Lee and Grossman 2006, Ginda *et al.* 2013, Broedersz *et al.* 2014). In some species like *C. glutamicum* or *C. crescentus*, the segregation system is composed of orthologues of ParA, ParB and *parS* which have highly conserved functions. ParA, a walker type ATPase, drives the segregation process of the replicated chromosomes. ParB is a DNA-binding protein that is recruited to inverted repeats, called *parS* sites, on the chromosome where it forms a nucleoprotein complex (Jakimowicz *et al.* 2002). The *parS* sites are located near the origin of replication (*oriC*) region. The ParAB*S* system from *C. glutamicum* was previously characterized in our lab and the subcellular localization of ParB

was observed near the cell poles (Donovan *et al.* 2010). Moreover, a genomic insertion of a *tetO* array near the origin region was also localized near the cell poles, indicating a polar orientation of the *parS*-*oriC* region in *C. glutamicum* (Donovan *et al.* 2010). These observations led to the idea that polar tethering of the chromosomes could be achieved by protein-protein interaction. A similar situation was observed in *C. crescentus*, where the *oriC* region is tethered to the cell poles via interaction of the ParB-*parS* nucleoprotein complex with the polar scaffold protein PopZ (Viollier *et al.* 2004, Bowman *et al.* 2008). In *E. coli*, however, the *oriC* region of the chromosome is orientated to a midcell position. Shortly after initiation of chromosome replication, the template and replicated *oriC* region are partitioned to the ¼ and ¾ position of the cell. These subcellular locations mark the midcell of the future daughter cell (Gordon *et al.* 1997). In *B. subtilis*, the *oriC* is also located at a midcell position. When replication has been initiated, a central replisome complex stays at midcell whereas the *oriC* regions are pushed symmetrically towards the prospective daughter cells. After division, the *oriC* regions are again located in a midcell region (Lin and Grossman 1998, Donovan and Bramkamp 2014).

In *C. glutamicum* the *oriC* region of the chromosome is localized at one of the cell poles. As replication begins and the *oriC* region is duplicated, the daughter *oriC* is partitioned to the opposite cell pole, where it appears to be tethered in place. We speculated that the polar localized, apical growth determinant DivIVA might play a role in polar anchoring of the chromosomes (Letek *et al.* 2009, Lenarcic *et al.* 2009) and that protein-protein interactions were required for correct orientation of the chromosome. For the investigation of protein-protein interaction we established an assay where the proteins of interest are fused to fluorophores and are heterologously expressed in *E. coli* BL21 cells. The vector pETDuet-1 (Novagen) is ideal for this purpose as it harbors two multiple cloning sites (MCS) that allow simultaneous co-expression of the protein fusions. Each MCS has its own T7 promoter sequence. First, the fusion proteins were expressed individually. ParB localized to the nucleoid, likely upon unspecific binding to the DNA, as *E. coli* chromosomes do not contain a perfect *parS* consensus sequences (chapter 2.1). DivIVA localized to the cell poles and septa, likewise in its natural host. Upon co-expression, DivIVA recruited ParB to the cell poles. The fact that *E. coli* serves as heterologous reaction vessel excludes the possibility that further proteins might be involved in co-localization and thus gives hint to direct physical interaction between DivIVA and ParB. The assay was also applied to homologues of different species, demonstrating that DivIVA-ParB interaction is a common feature in Actinobacteria. To map the interactions sites of DivIVA and ParB, mutant proteins were analyzed, showing that a conserved arginine residue in ParB (R21) is involved in DivIVA interaction. Mutation of the conserved arginine to an alanine (R21A) abolished the interaction between ParB and

DivIVA. Indeed, when ParBR21A was expressed extra-chromosomally in *C. glutamicum* in a Δ*parB* background, the gross segregation defects of the Δ*parB* mutant phenotype was not completely complemented (Donovan *et al.* 2010). In addition, the localization of the ParB foci, which can be used as a proxy for the localization of the *oriC* regions, was altered with a significant proportion of cells completely lacking polar localized ParB foci. Mutagenesis of DivIVA revealed that a central region of 154 AAs (AA 144-298) plays a role in ParB interaction (chapter 2.1). Although direct interaction between DivIVA and ParB could be demonstrated, these two proteins alone are not sufficient for chromosome segregation. In *C. glutamicum*, also ParA is required for proper functioning of segregation, as highlighted in a *parA* deletion mutant with mislocalized ParB foci, altered phenotypes and DNA free cells (Donovan *et al.* 2010).

Besides the spatial aspect of DivIVA mediated polar tethering of the chromosomes, also the temporal regulation of division and growth is imperative for the production of viable offspring. In other rod-shaped bacteria, such as *E. coli* and *B. subtilis*, the Min system and nucleoid occlusion (Noc) play a role in temporal and spatial regulation of cytokinesis. *C. glutamicum*, and other members of the Actinobacteria phylum, lack a Min system and a nucleoid occlusion system has not been identified to date. However, unlike *E. coli* and *B. subtilis*, *C. glutamicum* does not always divide precisely at midcell. Interestingly, mutation of the Par system not only altered the organization of the chromosome but also gave rise to spatial and temporal defects in cell division as well as altered polar cell growth (Donovan *et al.* 2013). Deletion of *parB* led to morphological alterations that appeared to be a consequence of reduced polar growth. It could be speculated that docking of the *oriC* at the cell poles through ParB-DivIVA interactions stimulates polar growth. It was also observed that *C. glutamicum* Δ*parB* mutant cells tend to divide before nucleoid segregation has been fulfilled, resulting in guillotined chromosomes (Donovan *et al.* 2013). Taken together, this suggests an involvement of chromosome segregation in spatial and temporal regulation of cell division and growth. Division site selection is largely altered and growth rates and generation times are more variable in mutants defective of chromosome segregation. It is thus believed that this process determines time and space for cell division in *C. glutamicum* (Donovan *et al.* 2013).

3.2 RodA and lipid II transport

Like many rod-shaped bacteria, *C. glutamicum* harbors two machineries for cell wall growth: one for septal division growth and one for polar elongation growth. Septal division growth is fulfilled by gene products that are encoded in the *fts* operon, which is conserved among

bacterial species (Bi and Lutkenhaus 1991, Goehring and Beckwith 2005). The prokaryotic tubulin homologue FtsZ, which polymerizes to a ring-like structure, called the Z-ring, spatially regulates the cell growth machinery at the site of septation. Penicillin-binding protein FtsI (PBP3) directly interacts with FtsZ and incorporates new PG precursors into the new cell wall (Weiss *et al.* 1997, Datta *et al.* 2006). The essential lipid II flippase FtsW also interacts with FtsZ and fulfills lipid II translocation over the membrane (Datta *et al.* 2002, Mohammadi *et al.* 2011). Inactivated FtsI was shown to inhibit Z-ring constriction and FtsW plays a role in FtsZ ring stabilization, implicating the essentiality of these three components among others for proper cell division (Pogliano *et al.* 1997, Boyle *et al.* 1997).

During cell elongation in *C. glutamicum*, intercalation of nascent PG subunits is carried out by polar localized growth machinery. This machinery is spatially regulated by DivIVA, which recognizes negative membrane curvature and self-localizes to the cell poles (Letek *et al.* 2008b, Lenarcic *et al.* 2009). Three PBPs were identified to be involved in elongation growth, namely PBP1a, PBP1b and PBP2b (Valbuena *et al.* 2007). None of the three PBPs turned out to be essential and thus seem to be redundant. The second lipid II flippase RodA is also a member of the polar elongation complex. Subcellular localization of RodA was observed at the cell poles and, at a late stage of the cell cycle, at the division site. A markerless deletion of *rodA* had effects on cell morphology and growth, however, to our surprise, not on viability and polar cell growth. This observation raised the question of how polar growth is maintained. To answer this, we first performed a vancomycin fluorescent (van-FL) staining to visualize extracellular lipid II. It turned out that WT cells were stained exclusively at the poles and septa, as previously shown (Letek *et al.* 2008b), whereas Δ*rodA* mutant cells were stained homogenously around their entire envelope, giving first hints for lateral movement of septal FtsW flipped lipid II that subsequently migrates towards the cell pole, where the polar PBPs are located. In addition, we measured a strong increase in van-FL fluorescence implicating higher lipid II amounts on the cell surface. To support the van-FL observations, both WT and Δ*rodA* mutant cells were treated with nisin. Nisin is a lantibiotic (lantibiotics are peptide antibiotics that contain the polycyclic amino acid lanthionine) that forms a distinct 8:4 complex with lipid II and penetrates the cell membrane leading cell death (Breukink and de Kruijff 2006). Growth experiments and phenotypic analysis revealed reduced sensitivity of the Δ*rodA* mutant compared to WT. We interpret this with reduced complex formation due to lower local density of lipid II on the surface of Δ*rodA* mutant cells and thus less reactivity of nisin. Finally, we measured membrane potentials of both WT and *rodA* mutant strains at different nisin concentrations to confirm penetration of the membrane and depolarization upon proton efflux. At sublethal nisin concentrations (20 µg/ml), we observed sustained potential in mutant cells compared to WT, indicating less pore formation. Taken together,

these results indicate extracellular existence and lateral movement of lipid II from the septum to the cell poles for polar growth. Our findings are in contrast to the observations that were made in *B. subtilis*, where the localization of MreB mediated PG synthesis depends on substrate (lipid II) availability (Lages *et al.* 2013).

Although it has never been shown, RodA is believed to fulfil lipid II translocation over membranes, similar to its homologue FtsW. FtsW has been identified to be necessary and sufficient for lipid II transport *in vitro* (Mohammadi *et al.* 2011). Membrane vesicles were purified from *E. coli* cells and fluorescently labelled lipid II (NBD-lipid II) was introduced. In vesicles from cells overexpressing FtsW, lipid II translocation from the inner to the outer leaflet was induced and rendered accessible to fluorescently labelled vancomycin (TMR-vancomycin) as detected by FRET. Similar results were obtained from vesicles derived from strains expressing FtsW from other organisms, signifying a species independent function. On the contrary, vesicles from a FtsW depletion strain showed reduced lipid II transport, implying the requirement of FtsW for NBD-lipid II translocation (Mohammadi *et al.* 2011). The residual transport activity that was observed was likely due to the presence of RodA which is supposed to have the same function. RodA and FtsW from *E. coli* share 31.9% identity of a 320 AA overlap and have similar topologies, according to a topology prediction with TMHMM (Ikeda *et al.* 1989, Arnold *et al.* 2006). The specificity of FtsW mediated lipid II transport could be mapped to two AAs in transmembrane domain four (Mohammadi *et al.* 2014).

Interestingly, a recent study contradicts the literature of the past decades, stating that MurJ and not FtsW (and possibly RodA) is responsible for lipid II transport (Sham *et al.* 2014). MurJ had been identified previously in a bioinformatics approach as a possible lipid II flippase in *E. coli* (Ruiz 2008). It is an inner membrane protein and member of the MOP (multidrug/oligo-saccharidyl-lipid/polysaccharide) exporter family. The gene is located within the *mur* operon which is well characterized for cell wall precursor synthesis (Mengin-Lecreulx *et al.* 1989). Whereas MurJ homologues from *E. coli* and *Burkholderia cenocepacia* are essential for viability (Sham *et al.* 2014, Mohamed and Valvano 2014), the one from *B. subtilis* is not necessary for growth and thus claimed as not serving as lipid II flippase in this organism (Fay and Dworkin 2009). *C. glutamicum* also has a MurJ homologue (cg3419) which has not been characterized, yet. It would be interesting to determine the localization, role, interactome and essentiality of MurJ in *C. glutamicum* with regard to its spatiotemporal regulation.

3.3 A robust protein-protein interaction assay by means of FRET

In order to rapidly analyze and quantify protein-protein interactions, the heterologous *E. coli* interaction assay was optimized for a FRET (fluorescence resonance energy transfer) based assay. FRET is based on the transfer of emitted fluorescence energy of the donor fluorophore (CFP) that can excite the acceptor fluorophore (YFP). If the molecules of interest interact, the donor chromophore transfers energy to the acceptor upon excitation with light of the wavelength that corresponds to the absorption maximum of the donor fluorophore. In the case of a positive FRET, excitation of CFP leads to emission of YFP fluorescence. The most important prerequisite for the generation of a FRET signal for the determination of protein-protein interaction is that the fluorophores are no further than 10 nm away, which is usually fulfilled in case of a direct physical interaction of the proteins of interest. In addition, the fluorophores have to be a FRET pair, meaning that the emission wavelength of the donor and excitation wavelength of the acceptor are in the same range. FRET was measured in a plate reader and an emission spectrum was monitored. The resulting spectra had usually two maxima that reflect the emission wavelengths of the two fluorophores.

The proteins of interest were individually tagged to one of the FRET compatible fluorophores, eCFP and eYFP (Sourjik and Berg 2002), and transformed into *E. coli* BL21 DE3. After cultivation in LB medium and IPTG-induced protein expression for one hour, FRET could be measured after one optional washing step in saline (0.9% NaCl solution). With this assay, we identified interaction between DivIVA and RodA (chapter 2.2). The ratios between the two maxima of the emission spectrum after co-expression (named R_{CY}) were calculated and used for quantification. To demonstrate the robustness of this FRET assay, several positive interaction partners as well as negative controls were tested (chapter 2.3).

The ε subunit of the *B. subtilis* F_0F_1-ATP synthase sandwiched between a FERT pair served as a positive control when expressed from plasmid pRSETB_AT1.03 (Imamura et al. 2009). The ε subunit binds ATP which leads to a conformational change that brings the FRET pair in close contact. As a hydrolysis mutant variant of the ε subunit is used, the conformational change of the ε subunit is long-lived and can be measured. Upon expression in *E. coli*, a clear FRET signal was generated due to the presence of physiological ATP. We also included protein interaction between DivIVA and ParB, as an example for a protein pair that consists of one soluble and one membrane attached protein. Together with the non-interacting ParB mutant (ParBR21A), we obtained a nice distinguishable positive/negative FRET pair. As negative controls, we included several integral membrane proteins, such as FtsW or BetP, which were not supposed to interact with DivIVA and indeed did not generate a FRET signal. Of note, a slight energy transfer could be observed here as well. This is due

to random approximation of the fluorophores when both proteins of interest, they are tagged to, have the same topology. This is for example the case for a protein pair with two trans-membrane proteins, or for a protein pair with one transmembrane and one membrane attached protein. DivIVA is one such membrane attached protein and therefore a slight FRET signal was observed, when expressed together with a trans-membrane protein. Nevertheless, this slight FRET signal can easily be distinguished from the FRET that was obtained from protein-protein interaction. All ratios that were calculated had distinctive values and could be divided into 4 groups that were characterized as I) CFP fluorescence only, no interaction ($R_{CY} < 0.9$); II) coalescence due to approximation of fluorophores, however without co-localization or interaction ($0.9 < R_{CY} < 1.1$); III) co-localization upon protein interaction ($1.1 < R_{CY} < 1.3$) and IV) YFP fluorescence only ($R_{CY} > 1.3$) (chapter 2.3). A striking control experiment was performed upon expression of untagged fluorophores. When observed under the microscope they both localized in the cytoplasm, however, a FRET signal could not be observed (chapter 2.3, supplementary material). This result clearly shows that apparent co-localization of proteins under normal epifluorescence microscopy is certainly not a reliable method to investigate protein-protein interaction or to interpret it as such. A positive FRET signal, however, is only generated upon direct protein-protein interactions with distances below 10 nm between the fluorophores. The distances between the fluorophores of the cytosolic fluorophores were significantly longer. Altogether, FRET serves as a robust and reliable assay and can be considered as decisive improvement of the synthetic *in vivo* system.

3.4 DivIVA spatially regulates the polar cell growth machinery

The polar self-localization of various DivIVA homologs has been extensively investigated in the past years (Muchova *et al.* 2002, Lenarcic *et al.* 2009, Oliva *et al.* 2010). In *C. glutamicum* DivIVA has been proposed to play a role in polar elongation (Letek *et al.* 2008b). To date, however, the exact role has not been demonstrated. A weak interaction of DivIVA with PBP1a was found in a bacterial-two-hybrid interaction assay, but the interaction was not characterized or further investigated (Valbuena *et al.* 2007).

We set out to investigate the role of DivIVA in polar cell growth, which was likely due to protein-protein interaction(s) between DivIVA and yet unknown protein(s), similar to the interaction between DivIVA and ParB for chromosome segregation. We performed co-localization studies with the described FRET assay and identified direct physical interaction between DivIVA and the lipid II flippase RodA (chapter 2.2). To further characterize this interaction, several truncation and point mutants of both proteins were generated and FRET

was measured (chapter 2.3). A lysine residue in the N-terminal domain (K20) of DivIVA was found to be essential for interaction with RodA. Loss of charge by mutation of the lysine to alanine (K20A), glycine (K20G) and isoleucine (K20I) abolished interaction with RodA. Mutation to an arginine (K20R), however, restored interaction, implicating that a positive charge is required for RodA interaction. All DivIVA mutant variants localized to the cell poles and division septa similar to the wild type protein, suggesting that K20 is not involved in membrane binding. The lysine K20 in *C. glutamicum* DivIVA is conserved in Actinobacteria, such as *C. glutamicum*, *M. tuberculosis* or *S. coelicolor,* suggesting that is must have a distinct role in polar elongation growth. Lateral growing firmicutes, such as *B. subtilis* or *L. monocytogenes,* have an exposed phenylalanine at the relevant position (F17), which is involved in membrane attachment (Oliva *et al.* 2010) (see alignment in chapter 2.3, figure 1). If K20 of DivIVA is responsible for RodA interaction, then how does $DivIVA_{Cgl}$ attach to the membrane? One possible idea is the involvement of further hydrophobic AA residues that are located in the tip region, such as isoleucine I18. To test this idea we mutated I18 to an aspartate (I18D) and a phenylalanine (I18F). It turned out that I18D had slight defects in membrane binding whereas mutant I18F, which carries a hydrophobic phenylalanine residue, had a similar binding capacity compared to WT DivIVA. Altogether, interaction of DivIVA with RodA requires a positively charged residue at position 20 (K20) of DivIVA, while membrane attachment is fulfilled by nearby located AAs in *C. glutamicum* DivIVA.

The interaction site of RodA was mapped to its cytoplasmic C-terminus, in particular to the last ten AAs (AAs 432-451: MSKQASEVAA). Truncation (ΔC10) as well as single and double point mutants (S433G/S437G, K434G, Q435G) revealed that interaction of RodA with DivIVA was weakened. When all ten AAs were mutated to AAs with similar functional groups or charges (mutC10: AVRNGIADGG), interaction was sustained, implicating an ionic charge-based protein interaction between RodA and DivIVA.

The interaction of DivIVA and RodA was corroborated from subcellular localization studies in *C. glutamicum*, where RodA foci were found only together with DivIVA at approx. 60% of the cell poles and 30% of the septa when expressed as fluorescent protein fusions. DivIVA-independent RodA foci were not observed, neither at the poles nor at the septum, indicating that RodA localization depends on DivIVA.

To date, no crystal structures of RodA or FtsW exist and structure modeling of both proteins did not provide evidence for an interaction topology. Moreover, the crystal structure of the DivIVA N-terminus has only been resolved for *B. subtilis* DivIVA. The structure of *C. glutamicum* DivIVA is still unknown.

A recent study claims that addition of new cell wall material in *M. tuberculosis* does not occur at the DivIVA-marked tip but is directed to a subpolar location (Meniche *et al.* 2014). This is achieved by interaction of DivIVA (Wag31) with enzymes that are involved in early steps of precursor synthesis, AccA3 and AccD5, both members of the acyl-CoA carboxylase (ACC) complex together with AccD4. This Wag31-associated complex is responsible for the synthesis and activation of 2-carboxyacyl-CoA (Gande *et al.* 2007). In this reaction cascade, the polyketid synthase Pks13 catalyzes the condensation of 2-carboxyacyl-CoA with activated meromycoloyl-AMP, yielding MA after reduction by CmrA (Portevin *et al.* 2004). Although spatially regulated by Wag31, here it seems that cell wall synthesis is excluded from the tip and directed to the border between the cylindrical body and the poles where new cell walls are added circumferentially in a manner that is analogous to lateral cell growth like in *B. subtilis* or *E. coli* (Meniche *et al.* 2014). We tested the idea of subpolar addition of new cell walls in *C. glutamicum*. Using a similar staining method as mentioned in the above study, composed of an Az-D-Ala treatment in combination with DBCO-carboxyrhodamine to visualize nascent PG, our results show that this is not the case in *C. glutamicum* (chapter 2.4). Moreover, we applied the staining to *Mycobacterium phlei*. Likewise, *M. phlei* cells did not show subpolar PG insertion (K. Schubert, personnel communication). The advantage of this staining assay is that Az-D-Ala treatment is not lethal to the cells, compared to a vancomycin-FL staining. We therefore applied a pulse labelling approach, where we first stained Az-D-Ala modified cells with a green fluorescent dye (DBCO-carboxyrhodamine), prior to further incubation and subsequent staining with a second, red fluorescent dye (DBCO-TexasRed). Using fluorescence microscopy we could show that the first staining procedure (green) exclusively labelled the cell poles. After further incubation and a second staining procedure, we saw that the extended cell poles were labelled exclusively with the second dye (red), whereas the subpolar area remained labelled in green (see chapter 2.4, figure 3B).

Unlike to the other components of the polar elongation machinery, such as RodA and the PBPs, DivIVA cannot be deleted in *C. glutamicum*, likely due to its versatility and involvement in further processes such as chromosome tethering.

3.5 Polar cell growth inhibition in *C. glutamicum*

We applied our knowledge in polar cell growth and spatiotemporal cell cycle regulation to investigate stress response mechanisms of EMB and BTZ043. Both antibiotics target enzymes of the AG synthesis pathway: BTZ043 the decaprenyl-phosphoryl-β-D-ribofuranose 2'-epimerase DprE1 for DPR isomerization to DPX and EMB the arabinosyltransferases

EmbABC during processive addition of arabinofuranosyl residues to the arabinan chains (Escuyer *et al.* 2001, Alderwick *et al.* 2005, Goude *et al.* 2009, Makarov *et al.* 2009) (Figure 6 and Figure 7). Since *Mycobacteria* require their mycoloyl-arabinogalactan layer for growth and survival, they have a high susceptibility to both antibiotics, which makes it difficult to study AB effects and response mechanisms before the cells die. Therefore, we used *C. glutamicum* as model organism as it is closely related to *Mycobacteria* but whose mycolic acid layer is not essential for viability.

Both antibiotics that were investigated showed similar phenotypic effects with shorter and thicker cells. EMB had a significant effect on growth and final OD, whereas the effect of BTZ043 on cell growth was less pronounced (chapter 2.4). Using a chromosomal DivIVA-mCherry insertion we observed a drastic increase in DivIVA protein level after AB treatment. RT-qPCR analysis revealed that the DivIVA transcription level was not increased. This suggests that due to the inhibition of growth, the progressive dilution of the constitutively expressed DivIVA protein was reduced, resulting in excessive DivIVA. Cell staining assays with fluorescent DHPE, a mycolic acid stain, and with Az-D-Ala in combination with DBCO-carboxyrhodamine for nascent cell wall labelling showed that both ABs exclusively affect polar cell wall synthesis. Septal cell wall synthesis that occurs during division was not targeted. Cell wall analysis of purified and hydrolyzed cell walls revealed that both ABs alter the composition of *C. glutamicum* cell wall. Compared to untreated cells, AB treated cells had significantly less arabinose and no rhamnose, as shown by thin layer chromatography (chapter 2.4 figure 4). Rhamnose is part of the linker molecule that connects AG with the PG sacculus and lack of rhamnose implicates that AG and MA are released from the PG frame. Electron microscopy images revealed that the cell surface of *C. glutamicum* cells was significantly altered after AB treatment, corroborating alterations in the composition of the cell wall.

The observation that both antibiotics also inhibited PG synthesis raised the question of how PG and AG synthesis are connected. Since BTZ043 inhibits procession of the decaprenyl-carrying DPR intermediate (Figure 6), we set out to test if irreversible consumption of decaprenyl pyrophosphate is one reason for the inhibition of PG synthesis. Decaprenyl phosphate is the versatile carrier molecule for several translocation processes over the membrane, like for lipid II, LAM and teichoic acids and thus one connection hub between AG and PG synthesis (Lazarevic and Karamata 1995, Mohammadi *et al.* 2011). Besides DPP, some species utilize undecaprenyl phosphate as a lipid carrier. Whereas *M. tuberculosis* primarily utilizes DPP, *M. smegmatis, S. aureus* and *E. coli* utilize undecaprenyl phosphate (Higashi *et al.* 1970, Crick *et al.* 2000, Mahapatra *et al.* 2005). As the differences of both carrier molecules are insignificant, we designate the carrier molecule that is present in *C.*

glutamicum as decaprenyl phosphate. If irreversible DPP consumption led to shortage of such for lipid II translocation, this would explain why PG synthesis is affected by BTZ. To test this idea, we overexpressed the DPP synthetase UppS1. It turned out that UppS1 overexpression counteracts BTZ inhibition, simply by filling of the DPP pool and thus re-enabling translocation of lipid II, and possibly teichoic acids. From these results we can conclude a side effect of BTZ043 inhibition, which is the constitutive withdrawing of DPP, resulting in shortage of such for other flipping processes over the membrane. The relation between UppS level and AB susceptibility was previously observed in *B. subtilis*, where reduced UppS levels caused increased susceptibility to cell wall acting antibiotics (Lee and Helmann 2013). Unlike BTZ043 inhibition, UppS1 overexpression did not complement EMB inhibition of PG growth, suggesting that EMB mediated PG growth inhibition is not due to DPP consumption. A possible alternative is the existence of a large cell wall synthetic super complex where the components are highly interdependent and inhibition of one component leads to inhibition of the whole synthetic complex.

Altogether, we conclude that polar cell wall growth is not essential for viability in *C. glutamicum*, as neither gene deletions, such as *rodA,* nor AB treatment (EMB, BTZ) result in cell death or extinction of the population. Most likely, the septal cell wall growth machinery is capable of taking over cell growth and, to an extent, ensures that viability is preserved.

When AB mediated polar growth inhibition is stopped before cell death, e.g. in an incomplete AB therapy, *C. glutamicum* cells proceed an impressively effective method to recover from AB stress. Excessive protein amounts, for instance, are collected in one daughter cell, to enable normal growth for the other daughter cell (chapter 2.4). The asymmetric distribution of aggregated protein happened in 51% of the cases already from the first generation on, and all cell lines were able to recover at least within 3 generations. This asymmetric recovery behavior highlights another interesting example for cellular asymmetries in *C. glutamicum*, such as division site selection and polar growth speed.

3.6 A revised model of spatial cell cycle regulation

Based on our results on spatial cell cycle control we propose a new model that describes the connection between chromosome segregation and elongation growth, as well as temporal regulation of cell division (Figure 8). In an early stage of the cell cycle, shortly after a previous division event, the chromosome is condensed and the origin is located close to the original (old, right) cell pole (A). As the cell elongates, chromosome replication is initiated. The replicated daughter *oriC* is subsequently segregated towards the opposite (new, left) pole. At that stage, the old cell pole grows faster than the new pole (chapter 2.2). A possible

explanation for this difference in growth speed is that the new cell pole goes through a rebuilding phase where the divisome is disassembled and a new elongasome is made. In addition, it might be possible that a regulatory system is employed that stalls elongation growth of the new pole until the new *oriC*-ParB complex (yellow dot) has reached the new cell pole.

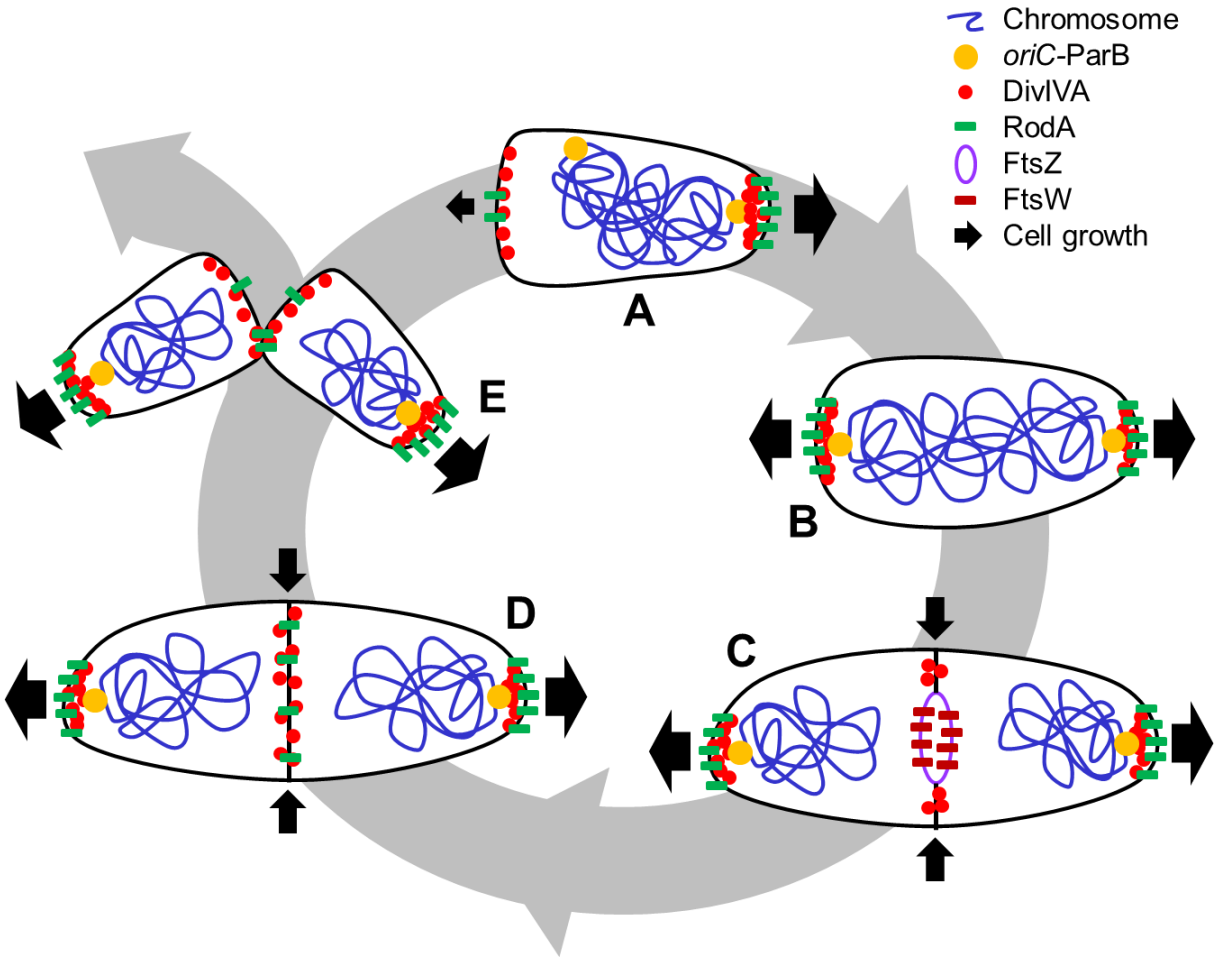

Figure 8: Proposed revised model for chromosome segregation, elongation growth and cell division in *C. glutamicum*. After division, the cells elongate from the poles at different growth speed, in which the old pole grows faster than the new pole (A). In the meantime, chromosome replication and segregation proceeds (B). After segregation, the new division site is marked within the midcell gap between the two nucleoids (C). FtsZ ring constriction initiates the formation of the new septum (D). DivIVA localizes to the division septum and recruits further proteins, such as RodA, to prepare the new polar elongation machinery. Finally, the cell divides giving rise to two fully equipped daughter cells (E).

When the replicated nucleoid arrives at the new pole, it is tethered via DivIVA-ParB interaction (chapter 2.1). ParB binds to *parS* sites that are located on the chromosome near the *ori* region (B). It remains unclear, whether nucleoid anchoring triggers elongation growth at the new pole in a way that both poles now grow with the same speed. As soon as the segregation process opens enough space at or close to midcell (C), septum formation and construction is carried out. The site of septation is previously defined by FtsZ, which forms a

ring-like structure, called the Z-ring. Although it is speculated that the chromosome itself, the organization of the chromosome or a factor associated with the chromosome might spatially influence division site selection, it is not yet clear if regulatory systems play a role. FtsZ lays the foundation of the division site and is necessary for recruitment of further division proteins that are also encoded in the *fts* operon. In *E. coli*, FtsA is required to target FtsZ to the membrane (Pichoff and Lutkenhaus 2002). Other division proteins are the septal lipid II flippase FtsW (Datta *et al.* 2002, Mohammadi *et al.* 2011) or penicillin-binding protein FtsI for septal PG formation (Weiss *et al.* 1997) and several regulator proteins such as EzrA, ZapA and SepF for positive or negative regulation of Z-ring formation. Since *C. glutamicum* lacks division site selection machineries such as Min or Noc, assembly of FtsZ does not always occurs precisely at midcell, however some regulatory system must exist given that the divisome assembles in the vicinity of the midcell region. When chromosome partitioning has been fulfilled, septum formation occurs in between the segregated nucleoids within a range of 20% of division length (chapter 2.2). Due to the formation of new negative membrane curvature at the septum (C-D), DivIVA is able to localize to the division site at a late stage (Gamba *et al.* 2009), where it recruits further proteins, such as RodA (chapter 2.2) or PBP1a (Valbuena *et al.* 2007), that are required for the formation of the new pole. Finally, cell division is completed, giving rise to two daughter cells (E). The fact that cell division does not always occur precisely at midcell has crucial consequences on the progeny. The daughter cells of each division event are not identical but differ in size. Moreover, recent observations showed that the two poles are not equally equipped with the entire elongation machinery. The old cell pole has approximately 2.1 ± 0.2 times more DivIVA than the new pole and also the RodA amount is significantly higher at the old cell pole, suggesting that the elongation machinery is not fully developed at the new pole. As a consequence, the two poles grow with different speed, with the old pole growing faster than the new pole (chapter 2.2). Another interesting asymmetry was observed on a population level, during recovery from EMB stress (chapter 2.4). Excessive DivIVA amounts were stored in a heterogenic manner in one daughter cell to enable the other daughter cell to exhibit normal growth and thus to set up a new intact population.

Altogether, we can conclude that *C. glutamicum* is a striking example for asymmetries during division and growth. In other species, such as *C. crescentus* for instance, asymmetric division is more obvious. In this organism cell division gives rise to two morphologically and functionally different cells, a swarmer cell and a stalked cell (Poindexter 1964, Shapiro *et al.* 2002). The stalked cell has a tubular stalk with an adhesive tip which allows adherence to surfaces. Moreover, it can undergo normal chromosome replication and cell division. The

swarmer cell has a flagellum which provides swimming motility for chemotaxis. However, it cannot undergo cell division, and has to differentiate into a stalked cell beforehand.

3.7 Summary and outlook

The involvement of *C. glutamicum* DivIVA in polar cell growth has been proposed several years ago but it was never investigated in detail. Consequently, the exact function of DivIVA was not clarified and possible interaction partners were not characterized.

A role of DivIVA in chromosome segregation has been suggested from the observation of the polar orientation of the *oriC*-ParB nucleoprotein complex in *C. glutamicum*. The polar determinant DivIVA was a good candidate for the involvement of *oriC*-ParB tethering to the cell poles.

In the present study we present our data about the identification and characterization of two functions of *C. glutamicum* DivIVA: its role in chromosome tethering via interaction with ParB and its role in polar cell growth via interaction with RodA. We mapped the sites of DivIVA required for interaction with ParB and RodA to see whether both proteins share or compete for the same interaction site of DivIVA. This hypothesis was questioned as possible connection knot between cell wall growth and chromosome segregation. It turned out that RodA and ParB do not share the same interaction site, suggesting that DivIVA has a modular character. Figure 9 gives an overview of the present DivIVA interactome of *C. glutamicum.*

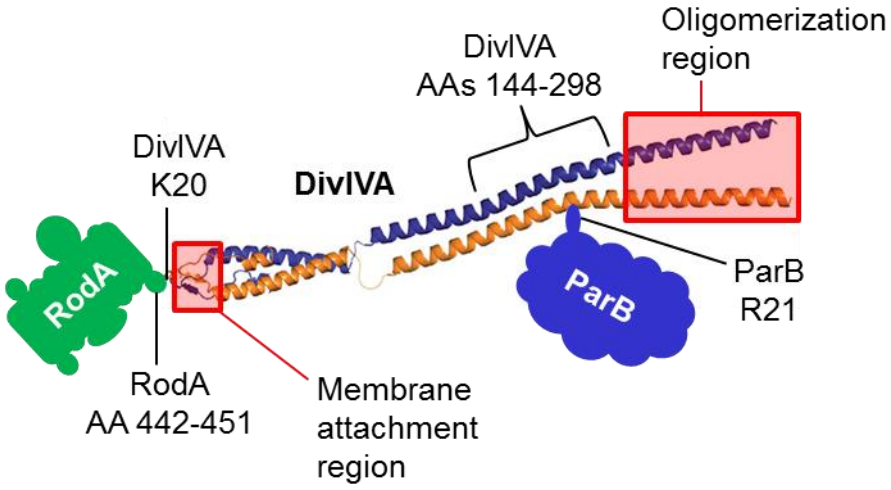

Figure 9: Model of DivIVA with its interaction partners RodA and ParB. RodA binds with its C-terminal end (AAs 432-451) to lysine residue K20 in DivIVA, which is presumably located at the tip of the loop. ParB binds with its arginine residue R21 to a middle region (AA 144-298) of DivIVA (Donovan *et al.* 2012). The DivIVA structure was adapted and modified from *B. subtilis* DivIVA (Oliva *et al.* 2010).

The DivIVA crystal structure in figure 9 has been adapted from *B. subtilis* DivIVA (Oliva *et al.* 2010). We presume a similar topology for $DivIVA_{Cgl}$, as it was predicted for the N-terminal domain from structure modeling with Swiss-Model (Arnold *et al.* 2006). DivIVA interacts via lysine residue K20 with the C-terminal end (AAs 442-451) of RodA. Moreover, a central region of DivIVA (AAs144-298) is required to interact with ParB, whereas arginine residue R21 of ParB is crucial for interaction with DivIVA. In addition, DivIVA has a strong oligomerization tendency and the ability to attach to membranes, with both characteristics dependent on specific domains. It has been shown for *B. subtilis* DivIVA that oligomerization is maintained by the C-terminal coiled-coil domain (Muchova *et al.* 2002), whereas membrane attachment requires phenylalanine residue F17 in the N-terminal domain (Oliva *et al.* 2010). Interestingly, *C. glutamicum* DivIVA has a lysine residue at the corresponding position which we have found to be crucial for RodA interaction. It is therefore likely that the mechanism of membrane attachment differs between DivIVA from *B. subtilis* and *C. glutamicum*. It would be interesting to further characterize *C. glutamicum* DivIVA in terms of membrane attachment and its topology, which could be responsible for a modular switch, for instance when ParB interaction enables RodA interaction to trigger polar growth (compare with Figure 8). Resolving the *C. glutamicum* crystal structure would be a first step towards this idea.

The asymmetry in cell division of *C. glutamicum* not only results in two non-equal daughter cells but also in asymmetry within each cell. The two cell poles of a new born cell grow with different speed, likely as a consequence from the poorly equipped elongation machinery at the new cell pole, where less protein amounts of the components of the elongation machinery, such as DivIVA and RodA, were observed. At a late stage in the cell cycle, however, both cell poles exhibit the same growth speed, most likely when the new *oriC*-ParB complex has arrived at the opposite pole and chromosome partitioning has been fulfilled. It could be possible that arrival of the segregated chromosomes at the opposite pole triggers growth speed of the new pole. Time lapse studies with fluorescently labelled *oriC*-ParB, DivIVA and with fluorescently stained cell walls to measure the growth speed of the cell poles could be applied to test this idea.

It would be a milestone in bacterial cell growth analysis to solve the mystery of lipid II translocation. Although plenty of work has been done to approach this task, the detailed mechanism is still unclear. A crystal structure of RodA or FtsW, ideally together with their substrate, lipid II, would provide a huge step in understanding the mechanism of lipid II translocation. A crystal structure of RodA would also give information about the topology and structure of the C-terminal region for interaction with DivIVA. Besides the polar lipid II flippase RodA and the septal lipid II flippase FtsW, a homologue of the recently characterized

lipid II flippase MurJ is also present in *C. glutamicum*. It would be interesting to analyze a possible redundancy of this flippase or, alternatively, a different and yet unknown function of *C. glutamicum* MurJ.

Several experiments that were performed in the context of the present study revealed that polar cell wall growth is not essential for viability in *C. glutamicum*. Neither the deletion of genes that encode for the polar growth machinery (*rodA*, for example) nor antibiotic inhibition of polar cell wall growth is mortal. However, deletion of *divIVA* is presumed to be lethal, likely due to its involvement in polar cell growth and chromosome segregation. Unlike *Corynebacteria*, *Mycobacteria* require polar cell wall growth for viability. Although the structure of the complex PG-AG-MA cell envelope is conserved, there must be a significant difference that causes the essentiality in *Mycobacteria* compared to *Corynebacteria*. Identification of this difference would open a new field for AB research.

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